AD_____

Award Number: DAMD17-00-1-0076

TITLE: Regulation of the Response to Radiotherapy and Hyperthermia in Prostate Cancer by the 26s Proteasome

PRINCIPAL INVESTIGATOR: William H. McBride, Ph.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles
Los Angeles, California 90095-1406

REPORT DATE: April 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

930 057

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	April 2002	Annual (1 Apr 01 - 31 Mar 02)

4. TITLE AND SUBTITLE
Regulation of the Response to Radiotherapy and Hyperthermia in Prostate Cancer by the 26s Proteasome

5. FUNDING NUMBERS
DAMD17-00-1-0076

6. AUTHOR(S)
William H. McBride, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California, Los Angeles
Los Angeles, California 90095-1406

E-Mail: mcbride@radonc.ucla.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

20020930 058

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The goal of this proposal was to quantify the extent to which human prostate cancer cells vary in their proteolytic proteasome-associated activities, and to evaluate the role of the proteasome in the response of prostate cancer cells to hyperthermia and/or radiotherapy. We have documented the variation in proteasome activity between prostate cancer cell lines and have begun to explore its basis. We have confirmed that both irradiation and hyperthermia inhibit proteasome activity, but by different mechanisms. The inhibition is very rapid and occurs even after very low dose exposure. We have shown that it occurs in cytosolic and nuclear proteasomes to the same extent, and that the proteasome is a direct redox-sensitive target for the effects of hyperthermia and irradiation. The extent of proteasome inhibition is always partial, but its effects are profound. It seems to serve as a sensor that leads to changes in expression of NF-κB, p53, p21, c-jun, and many other genes. The nature of these changes will depend upon the turnover rate of the proteins, but clearly proteasome inhibition is part of the cellular stress response. The result of prolonged proteasome inhibition is cell death by apoptosis. We believe that this occurs through a capase-independent pathway. The clinical implications of variation in proteasome function between tumors and the effects of therapy on this system are profound. Heat-induced proteasome inhibition is probably responsible for radiosensitization that is observed with this modality. One proteasome inhibitor is entering Phase III clinical trials for multiple myeloma, and our data is taken as evidence of its ability to radiosensitize and as justification for a clinical trial in combination therapy for prostate cancer that is planned. We have shown that proteasome inhibitors can affect multi-drug resistance mediated by mdr1 and that HIV proteases may serve as anti-cancer agents by virtue of their ability to inhibit the proteasome.

14. SUBJECT TERMS	15. NUMBER OF PAGES
radiotherapy, hyperthermia, proteasome, NF-kB, prostate cancer, anti-cancer agents, cellular stress response, apoptosis, myeloma	184
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Regulation of the Response to Radiotherapy and Hyperthermia in Prostate Cancer by the 26s Proteasome

Second Annual Report

Research Accomplishments to Date:

Attached are 10 papers that have been published, are in press, or submitted manuscripts and that deal with the topic of the grant. This report summarizes these findings.

Introduction:

The goal of the proposal was to investigate the novel hypothesis that the proteasome functions as a sensor for hyperthermia and/or irradiation and its response is important in determining the outcome of prostate cancer treatment. In an attached review ((1); Appendix), we discuss the recent evidence indicating critical roles for the 26s proteasome in selectively regulating many vital cell processes, including cell cycle progression, oxidative stress, DNA repair, and cell death. We also show that this system is frequently dysregulated in cancer, including prostate cancer.

This proposal is novel in that the roles of the proteasome in the biology and therapy of cancer have been the subject of very few investigations and there are almost no reports on how their activities might influence the response of prostate cancer to therapeutic intervention. We postulated that proteasome activity would vary between prostate cancer cell lines, that hyperthermia and/or radiation would influence proteasome activity, and that this would influence the expression of cell death/survival molecules following treatment in a manner that could determine therapeutic outcome. We further suggested that the 26s proteasome might represent a novel potential target for prostate cancer therapy; one that is, as yet, unexplored.

Progress will be considered under the headings of the original stated tasks.

Body of the Report:

Task 1. To quantify proteasome activity in human prostate cancer cells and its modification by hyperthermia and/or radiotherapy.

We have examined the proteasome-associated chymotrypsin-like, trypsin-like, and peptidylglutamyl-peptide hydrolyzing activity in human PC3, LnCaP, DU-145, and a newly isolated human prostate line LAPC4, as well as the murine prostate cancer model TRAMP cell lines C1 and C2. We have compared this with activity in over 12 other types of human tumors. The clear picture that has emerged is one of considerable variation in constitutive steady state proteasome activity between cancer cell lines, which is what we predicted. We believe that this variation is a reflection of the molecular phenotype of the cell, and as a result, determines tumor behavior and the response to therapy. For example, we have shown that interleukin (IL)-6 and IL-3, both of which can be constitutively produced by prostate cancer cells, can enhance proteasome activity and autocrine pathways could be responsible for some of the variation between cancer cell lines. We have begun to characterize the composition of the proteasome subunits in cells to determine if some of these are cytokine-induced. There is a subset of proteasomes that fall into this category. The importance of this subset is that it presents peptide antigens to the immune system that are different from those normally produced. In other words the nature of the proteasome determines, not only the level of activity, but also the cleavage sites and recognition of the tumor by the immune system. While this will be the subject of a future grant application, in this period these techniques will be used to determine if all proteasomes respond to heta and/or irradiation in the same way.

The response of PC-3, LnCaP, and DU-145 to heat has been examined and this work has been submitted for publication (Appendix). Hyperthermia at 42 or 44 degrees for 30 minutes decreased 26s proteasome activity to about 40% of untreated control cells, as assessed by proteolysis of specific fluorogenic substrates (chymotrypsin-like activity was decreased to 36.2 ± 3% (PC-3), 33.4 ± 8.4% (DU-145) and 45 ± 3.4% (LnCaP)). The response was very rapid, if not immediate and is clearly an important part of the stress response of cells to hyperthermia. We have shown that this response leads to stabilization of IκBα, which is known to be degraded through the proteasome, with a resultant decrease in constitutive and heat-induced NF-κB. Since NF-κB is a survival factor for many cell types, the consequence was apoptosis and radiosensitization of the cell lines. In fact, we believe that this provides and explanation for the long-observed phenomenon of heat-induced radiosensitization.

Inhibitor studies showed that 26s proteasome activity was independent of transcriptional activity. Interestingly, 20s proteasome activity was unaffected. This suggests a) that blocking of the proteasome by oxidised proteins is unlikely to be the mechanism of inhibition b) that 19s regulatory subunits that feed proteins into the catalytic core are the target c) that mechanisms exist that allow regulatory control through proteasome inhibition and, simultaneously, allow oxidized and damaged proteines to be removed. The 20s proteasome is able to fulfil this function without assistance from the 26s proteasome.

Furthermore, and importantly, we have shown that heat downregulates expression of the androgen receptor (AR) on LNCaP cells. We hypothesize that this is brought about by dissolution of the AR-HSP90 complex. We have shown that the response to heat correlated with HSP90 induction, as assessed by PCR, in a temporal and temperature-related manner, but not with HSP70 or HSP27 expression. However, we have also shown that recombinant HSP90 blocks the activity of purified proteasomes, suggesting that this might be the physiololgical mechanism leading to proteasome blockade. The consequences of heat-induced AR downregulation have yet to be defined, but clearly this could have enormous importance as a mechanism for developing androgen independence in prostate cancer.

The effect of irradiation on proteasome activity has been assessed using the same prostate cancer cell lines. These studies have now been published (2, 3). We have confirmed that the proteasome is inordinately sensitive to very low doses of irradiation and that inhibition is almost immediate. The consequences of this finding for radiation-induced gene expression is profound. P53, mdm2, p21, IκBα, and numerous other immediate radiation-induced responses may be mediated, or at the very least influenced by, proteasome inhibition. This indeed may be the prime mechanism by which radiation and heat rapidly up-regulate protein expression in cells, leading to an adaptive response with transcriptional activation of new genetic programs.

In the published papers, we address an apparent paradox, which is that several groups of investigators, including ourselves, have shown that radiation induces NF-κB expression. Our hypothesis would anticipate that radiation-induced inhibition of proteasome activity would decrease expression. We have shown that at low doses inhibition is paramount and that only at high doses is inhibition overriden and NF-κB is induced, in part by pathways that are alternate pathways that involve tyrosine phosphorylation of IκBα. These finding provide a mechanism for the anti-inflammatory effects of low dose radiation that have been reported on numerous occasions and explain why radiation has long been used, especially in Europe, to treat inflammatory diseases.

Task 2. To identify pathways by which proteasome activity is altered in prostate cancer cells after hyperthermia and/or radiotherapy.

Because both radiation and heat alter the redox status of cells, we have investigated whether or not the proteasome is redox-sensitive. In fact, the 26s proteasome appears to be very sensitive to N-

acetyl cytseine, tempol, and glutathione treatment. Furthermore, at least the latter two free radical scavengers counteract radiation-induced proteasome inhibition. These studies have yet to be confirmed, but clearly indicate that the proteasome is a very sensitive redox sensor.

We have shown that irradiation of purified isolated proteasomes has the same level of effect on their function as does irradiation of cells followed by proteasome isolation. This suggests that the effects of irradiation are direct and not mediated by some soluble factor in the cell.

Furthermore we have shown that cytosolic and non-cytosolic (nuclear) extrcats are both affected in a cell. This is important because the spatial location of the proteasomes is being increasingly shown to dictate their substrates and functions.

As discussed above, we have identified NF-κB as a pathway that is clearly affected by heat- or radiation-induced proteasome inhibtion. Other targets are however likely and we have explored some of these. One possibility is that caspases are activated through inhibition of IAP degradation. Since the DNA repair enzyme DNA-PK is a substrate for caspase 3 we have investigated this as a possible pathway for heat-induced radiosensitization. Our data indicate that caspase 3 activation is not required for proteasome-dependent apoptosis and we are currently investigating non-caspase dependent pathways to apoptosis.

One of our hypotheses is that heat, but not radiation, caused inhibition of proteasome activity through activation of HSP90. In experiments designed to test this concept, we have prepared the vectors encoding anti-sense HSP90, sense HSP90, and HSP70. We have also prepared primers to examine the components of the proteasome produced by different prostate cancer cell lines and have developed assays for examination of 20s and 26s activity following gel electrophoresis. We have done this in conjunction with the development of glycerol gradient purification of 20s and 26s proteasomes.

We have also developed an in vivo assay with PC3 cells transfected to express Ub-GFP. Using this assay, we have shown that heat causes accumulation of GFP in individual cells, in keeping with its effects on proteasome function. Surprisingly, radiation does not have the same effect. We are not sure whether this is because different molecular targets are involved, or whether the assay is too insensitive to detect the radiation effects.

In the next year we will exploit these assays, which have taken a long time to develop. Dealing with macromolecular structures as large as 2 million daltons poses challenges that are not usually encountered with smaller proteins. A particular focus of the study will be on the cytokine-inducible proteasome subunits lmp7, lmp2, and Mec1. We have preliminary evidence that these are the major targets for radiation. If this is the case, it might explain the finding that we never reach 100% inhibition of proteasome function. It also would be very important from the perspective of the development of ant-tumor immunity. We have obtained mice that have these genes knocked out and intend to use these to examine the roles of these genes in presenting antigen in a TRAMP prostate tumor model. Again these studies will extend beyond this proposal, but serve to indicate the function the grant has served us in indicating new areas of research in prostate cancer.

Task 3. To determine the extent to which 26s proteasome activity determines the effectiveness of prostate cancer treatment by hyperthermia and/or radiotherapy.

We have now shown that hyperthermia inhibits proteasome degradation by prostate cancer cells and prevents radiation-induced NF-κB activation resulting in cell death by apoptosis. This mechanism could explain the radiosensitizing effect of heat given shortly prior to radiation treatment. Recently, we have shown that PS-341, a proteasome inhibitor, can radiosensitize prostate

tumors in vitro and in vivo (4, 5). This agent is in clinical trials alone in multiple myeloma and, in large part because of our findings, is being explored in combination with radiotherapy for treatment of prostate cancer.

We have also explored other agents that inhibit proteasome activity with a view to their clinical use as radiosensitizers. We have shown that sequinavir, which is used clinically in HIV treatment is a direct proteasome inhibitor that can cause radiosensitization and may be a useful agent for the treatment of cancer in combination therapy. The reason is that the HIV protease has evolved a similarity with the mammalian proteasome, which is presumably one of its mechanisms of pathogenicity.

We have also shown that there is similarity between multiple drug resistence gene product mdr1 has cross specificty with the 26s proteasome and that proteasome inhibtors may haelp in overcoming mdr resistance to therapy. This indicates the utility of using proteasome inhibitors in combination with chemotherapy.

Key Accomplishments:

- Further quantified proteasome function in prostate cancer cell lines
- Demonstrated inhibition of proteasome function in prostate cancer cells in response to hyperthermia
- Shown dependency of heat-induced proteasome inhibition on the 26S, but not 20S, subunit.
- Shown that heat-induced proteasome inhibition is very rapid and does not require protein synthesis.
- Shown temporal and temperature-dependent relationship between heating and HSP90 expression.
- Shown that HSP90 directly inhibits proteasome function.
- Shown heat stabilizes IκBα expression and decreases NF-κB expression.
- Shown that heat induces apoptosis and radiosensitization of prostate cancer cells and that this correlates with proteasome inhibition and loss of ability of radiation to activate NF-κB.
- Shown that radiation result in a very rapid loss of proteasome activty down to 50% of control values and that the proteasome is extremely sensitive to even very low doses of irradiation.
- Shown that radiation-induced proteasome inhibition results in increased IκBα expression and decreased NF-κB expression at low doses, but that this is circumvented at high doses, providing an explanation for the dual anti- and pro-inflammatory responses seen after irradiation.
- Shown that irradiation of cells and purified proteasomes result in the same level of proteasome inhibition, suggesting that the effects of irradiation are direct.
- Shown that radiation-induced proteasome inhibition appears to depend upon the type of proteasome in a cell and that not all proteasomes are inhibited.
- Shown that nuclear and cytoplasmic proteasomes are both inhibited by radiation.
- Established vectors for sense and anti-sense HSP 90 and HSP 70.
- Developed methodology for independently measuring 20S and 26S functional proteasome activity in gels.
- Developed methods for further purifying proteasomes on glycerol density gradients.
- Developed Ub-GFP stable transfectants that respond to proteasome inhibition by heat and other proteasome inhibitors, but not radiation.
- Demonstrated that the proteasome is redox sensitive and that free radical scavengers can prevent radiation-induced proteasome inhibition

- Ruled out our hypothesis that heat-induced inhibition of proteasome activity leads to caspase 3 activation, degradation of DNA-PKcs, and decreased DNA repair in response to ionizing radiation.
- Shown that caspase independent mechanisms are involved in apoptosis following proteasome inhibition
- Shown that the proteasome inhibitor PS341 causes radiosensitization of prostate cancer cells in vitro and in vivo.
- Shown that HIV protease inhibitors cause proteasome inhibition and may be of value as radiosensitizers for prostate cancer
- Shown that the proteasome and mdr1 have a degree of cross specificity and that proteasome inhibitors may be useful in eliminating multiple drug resistance.

Reportable Outcomes:

Pajonk, F. and W.H. McBride: Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. Radiother. Oncol. 59:203-212, 2001.

Pajonk, F. and W.H. McBride: The proteasome in cancer biology and treatment. Radiat. Res. 156:447-459, 2001.

Pervan, M., F. Pajonk, J-R. Sun, H.R. Withers and W.H. McBride: Molecular pathways that modify tumor radiation response. Amer. J. Clin. Onc. 24:481-485, 2001.

Pajonk, F., K. Riess, A. Sommer and W.H. McBride: N-acetyl-L-cysteine inhibits 26s proteasome function - implications for effects on NF-κB activation. Free Radical Biology and Medicine 32:536-543, 2002.

McBride, W.H., Pajonk, F., C-S. Chiang and J-R.: NF-kB, cytokines, proteasomes and low dose radiation exposure. Military Medicine 167:66-67, 2002.

Pervan, M., F. Pajonk, J-R. Sun, H.R. Withers, W.H. McBride: The proteasome inhibitor PS-341 is a potential radiosensitizer. In: Abstracts of Papers for the American Association for Cancer Research Annual Meeting, 2001.

Liao, Y.P. and W.H. McBride: Radiation affects antigen presentation by dendritic cells. In: Abstracts of Papers for the 49th Annual Meeting of the Radiation Research Society, 2002.

McBride, W.H., M. Pervan and F. Pajonk: Is the proteasome a redox-sensitive target for radiation and other stress signals? In: Abstracts of Papers for the 49th Annual Meeting of the Radiation Research Society, 2002.

Liao, Y-P., W.S. Meng and W.H. McBride: Antigen presentation by dendritic cells is affected after irradiation. In: Abstracts of Papers for the American Association for Cancer Research Annual Meeting, 2002, p. 480.

Pervan, M., K. Iwamoto and W.H. McBride: Proteasome function is affected by ionizing radiation. In: Abstracts of Papers for the American Association for Cancer Research Annual Meeting, 2002, p. 687.

Conclusions:

This study has identified the 26s proteasome as a direct redox-sensitive target for the effects of radiation and heat. This adds a new dimension to the mechanisms underlying cellular stress responses. Since such responses often determine the outcome of therapeutic intervention, the proteasome can serve as a target for therapeutic intervention, in particular in combination with hyperthermia, radiation therapy, and/or chemotherapy. In the last two years, appreciation of the subtle but powerful impact of the ubiquitin/proteasome system in the regulation of cell behavior has grown enormously. This grant has allowed us to participate in the growth of this field and to make novel and unique contributions to basic understanding of the role of the protreasome in prostate cancer that are being translated into cancer treatment. We are encouraged by these developments and foresee considerable progress in this area of research in the near future.

References:

1. Pajonk, F. and McBride, W. H. The proteasome in cancer biology and treatment. Radiat Res. *156:* 447-59, 2001.

2. Pajonk, F. and McBride, W. H. Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. Radiother. Oncol. *59:* 203-12, 2001.

3. McBride, W. H., Pajonk, F., Chiang, C. S., and Sun, J. R. NF-kappa B, cytokines, proteasomes, and low-dose radiation exposure. Mil. Med. *167:* 66-7, 2002.

4. Pervan, M., Pajonk, F., Sun, J.-R., Withers, H. R., and McBride, W. H. Molecular pathways that modify tumor radiation response. Amer. J. Clin. Onc. *24:* 481-485, 2001.

5. Pervan, M., Pajonk, F., Sun, J.-R., Withers, R. H., and McBride, W. H. The proteasome inhibitor PS-341 is a potential radiosensitizer. Proceedings of the American Association for Cancer Research Annual Meeting *42:* 666-667, 2001.

Appendices:

Copies of papers 1-4 listed under References, plus an additional 6 papers, are attached that have been published, are in press, or submitted manuscripts and that deal with the topic of the grant

APPENDICES

DAMD17-00-1-0076

WILLIAM H. McBRIDE, P.I.

RADIATION RESEARCH 156, 447–459 (2001)
0033-7587/01 $5.00
© 2001 by Radiation Research Society.
All rights of reproduction in any form reserved.

REVIEW

The Proteasome in Cancer Biology and Treatment

Frank Pajonk[a,1] and William H. McBride[b]

[a] *Department of Radiation Therapy, Radiological University Clinic, Freiburg, Germany; and* [b] *Department of Radiation Oncology, Experimental Division, UCLA School of Medicine, Los Angeles, California*

Pajonk, F. and McBride, W. H. The Proteasome in Cancer Biology and Treatment. *Radiat. Res.* 156, 447–459 (2001).

During the last 30 years, investigation of the transcriptional and translational mechanisms of gene regulation has been a major focus of molecular cancer biology. More recently, it has become evident that cancer-related mutations and cancer-related therapies also can affect post-translational processing of cellular proteins and that control exerted at this level can be critical in defining both the cancer phenotype and the response to therapeutic intervention. One post-translational mechanism that is receiving considerable attention is degradation of intracellular proteins through the multicatalytic 26S proteasome. This follows growing recognition of the fact that protein degradation is a well-regulated and selective process that can differentially control intracellular protein expression levels. The proteasome is responsible for the degradation of all short-lived proteins and 70–90% of all long-lived proteins, thereby regulating signal transduction through pathways involving factors such as AP1 and NFKB, and processes such as cell cycle progression and arrest, DNA transcription, DNA repair/misrepair, angiogenesis, apoptosis/survival, growth and development, and inflammation and immunity, as well as muscle wasting (e.g. in cachexia and sepsis). In this review, we discuss the potential involvement of the proteasome in both cancer biology and cancer treatment. © 2001 by Radiation Research Society

INTRODUCTION

Most potentially malignant tumor cells, like their normal counterparts, will die as they move outside the environment that nurtures them. Only a small number of cells in a small proportion of individuals will develop mutations that allow them to overcome these limitations and evolve into a malignant metastatic phenotype. This transition requires multiple mutations in multiple genes. It is logical to assume that targeting therapies to individual cancer-related genes or pathways will leave other pathways intact and allow at least a proportion of the tumor cells to survive. A superior tactic for cancer therapy would be to target molecules or complexes of molecules that control nodes where multiple pathways converge rather than molecules that function primarily in one pathway. One promising nodal regulator of cellular function is the proteasome.

For cells to perform their functions appropriately, they must control the rates of both synthesis and destruction of their proteins. During the last two decades, our understanding of the pathways involved in protein synthesis has grown enormously. Only recently has the importance of controlled proteolysis in dictating the level of protein expression, in functionally activating precursor proteins to their mature form, and in antigen processing been recognized. Importantly, proteolysis has also been shown to play a major role in the cellular response to stimulation. Here it has a major advantage over protein synthesis in the speed at which responses can be activated. Targeting pre-existing proteins for rapid cleavage is a common mechanism by which inhibitory proteins can be destroyed and signal transduction pathways can be activated. The 26S proteasome is the multimolecular structure most responsible for the controlled degradation of short- and long-lived proteins in eukaryote cells. This review emphasizes how cancer mutations affect molecular processing through this multicatalytic proteasome complex, the role of the functional alterations in the activity of the complex in cancer biology, and its potential as a target for therapeutic intervention in cancer treatment.

Proteolytic Systems

Proteolytic systems are highly conserved in eukaryotic cells (*1–6*). The major division is into lysosomal and nonlysosomal systems. While the former is dependent on cathepsin B, D, H and L, the latter can be subdivided into energy-independent and energy-dependent mechanisms. The major mediators of energy-independent proteolysis are calpain I and II. Most intracellular molecules, however, are degraded through the proteasome. For many years, proteasomes were considered as being involved only in normal housekeeping events that required protein turnover. More

[1] Author to whom correspondence should be addressed at Department of Radiation Therapy, Radiological University Clinic, Hugstetter Str. 55, 79106 Freiburg i. Brsg., Germany; e-mail: pajonk@uni-freiburg.de.

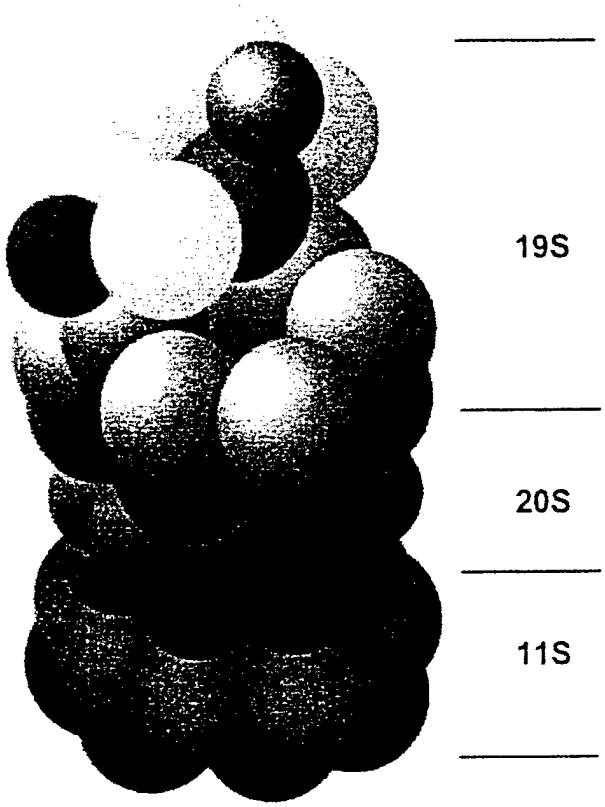


FIG. 1. Structure of a 19S–20S–11S hybrid proteasome as described in ref. (*23*). The 20S core unit consists of four rings of seven α and β units, respectively. The inner two rings of beta subunits form the catalytic center of the protease. Attached to both ends of the 20S core unit are 11S and 19S regulatory units. See the text for details.

recently, their roles in cell cycle progression, transcription and DNA repair, as well as in cellular responses to stress, have come to the fore. Several structural forms of proteasomes have evolved that have the same basic central core structure and that specialize in specific functions. The ATP- and ubiquitin-dependent 26S proteasome is responsible for controlled degradation of all short-lived proteins (*7*) and 70–90% of all long-lived proteins (*7, 8*). Non-ATP and non-ubiquitin proteasomal pathways are also present that tend to specialize in the removal of degraded proteins.

Structure and Function of Proteasomes

The proteasome consists of a large barrel-shaped 20S core unit of about 700 kDa that may have two 19S regulatory units (also known as PA700), or two 11S activator units (also known as PA28 or REG), or one 19S and one 11S unit (hybrid) attached to both ends (Fig. 1). The 19S regulator unit is formed from at least 18 different subunits. Some (Rpt1–6: human genes *PSMC1–6*) have ATPase activity and form a "base" to the 19S structure; others (Rpn1–12; human genes *PSMD1–12*) do not, and form the "lid." The 11S activator unit is formed from two subunits, α and β (PSME1 and PSME2, also known as PA28 α and β, or REG α and β). The 11S activator subunits share approximately 50% homology to a nuclear protein of unknown function, the Ki auto-antigen [recently identified as PSME3, also known as PA28γ (*9*)]. The 20S core is inactive unless it is activated by the 19S or 11S caps. Experimentally, it can be activated by SDS treatment.

The 20S core is a barrel-shaped structure of four protein complexes. The two outer rings have seven α subunits. The two inner two rings are built from seven β subunits, which form the catalytic sites. Proteins for degradation have to be unfolded and passed into the central core of the proteasome. Constitutive β subunits (β1, β2 and β5; human genes *PSMB6, PSMB7* and *PSMB5*) in the inner rings can be replaced by interferon γ-inducible subunits β1i, β2i, and β5i [LMP-2 (gene name *PSMB9*), MECL-1 (*PSMB10*), and LMP-7 (*PSMB8*), respectively] (*10–14*). The inducible subunits are coded in the class II region of the MHC locus. The 11S activator (PA28) subunits are also inducible by interferon γ. Structures containing inducible subunits are often called "immunosomes" because of their potency in processing antigen into peptides for immune presentation.

The 26S proteasome, which contains 19S regulatory subunits, is specialized in the recognition and destruction of proteins specifically targeted to it by the process of ubiquitinylation. A concerted cascade of three enzymes is generally involved in this process. First, ubiquitin, a 76-residue polypeptide found in all eukaryotic cells, is activated when its carboxy-terminal glycine is transformed into a high-energy thiolester intermediate by the ubiquitin-activating enzyme E1. A family of ubiquitin-conjugating (E2) enzymes conjugates ubiquitin to a diverse set of substrate recognition (E3) factors. These E3 ubiquitin ligases catalyze the last step, which is ligation of activated carboxy-terminal ubiquitin to amino groups of lysine residues in the targeted protein to form a polyubiquitin chain that is recognized by PSMD10 in the 19S regulatory unit. The E3 ligases are largely responsible for conferring specificity at the level of substrate recognition. Ligases belong to one of two major families, the HECT domain ligases (homologous to the E6 accessory protein carboxy terminus) and the RING finger ligases. E2 may also transfer activated ubiquitin directly to the substrate, and this may occur on the proteasome. Recognition of oxidized, damaged or excess proteins, at least in most cases, does not appear to require ubiquitinylation or proteasomes that contain the 19S regulator.

Proteasomal Function and the Immune System

Recognition of endogenously processed antigens by cytotoxic T lymphocytes requires epitopes to be presented in the context of major histocompatibility complex class I (MHC I) molecules. The α1 and α2 domains of MHC I molecules form a groove that holds peptides of 7–13 amino acids. While alternate nonproteasomal pathways exist for processing some antigens, most endogenously produced an-

tigens that generate cytotoxic lymphocytes are processed through the proteasome. The peptides are produced by the proteasome and delivered to nascent MHC I molecules in the lumen of the endoplasmic reticulum (ER) by specialized transporters that are associated with antigen processing (TAP) proteins. The affinity of binding of the peptides is determined by the nature of the interacting residues; binding requires the presence of one or more proline-rich anchor residues. Binding stabilizes the MHC I complex in association with the β2-microglobulin in a trimeric complex that moves to the cell surface so that the antigenic epitope can be presented to the cognate T cell [reviewed in refs. (*15–17*)]. The mechanism by which the proteasome cleaves proteins to generate products whose modal size, after N-terminus trimming, is a nonapeptide is not known. Immunoproteasomes formed under the influence of cytokines, in particular interferon γ, which is produced during activation of T cells specialized in cellular immunity, are particularly efficient at this cleavage process (*18–22*). The relationship between the 26S proteasome and the immunoproteasome in the processing of ubiquitinylated antigens is still uncertain, and hybrid proteasomes containing 19S and 11S subunits may be particularly effective in this situation.

Molecular Interactions with the Proteasome System and Evasion

In addition to indirect targeting of proteins for destruction through ubiquitinylation, two hybrid screens and other techniques have identified a number of proteins that interact directly with proteasomal subunits, in particular with those that form the 19S regulatory cap and that have ATPase activity (*23*). A number viral proteins such as Ad E1A, SV-40T, E7 and HVI-1 tat can interact in this manner, suggesting that viruses, including herpes simplex virus, cytomegalovirus, human papilloma virus, and human immunodeficiency virus type 1, have evolved strategies of replication and immune evasion that target the proteasome (*24–29*). Other interacting molecules are certain cell surface receptors, such as TNFRSF1A (TNFR1), TR, EGFR, ERα, RARA, and RXR. This may provide a very rapid mechanism by which cells can control responses to external stimuli. Demonstration of direct interactions with proteins involved in DNA repair (RAD23) and the cell cycle [CDC28, PSMD9 (p27)] indicate the importance of the proteasome in these processes. Finally, the proteasome is intimately linked with chaperones, such as HSP90, which play a role in unfolding proteins for insertion into the internal catalytic chamber and may also regulate proteasomal activity (*30*).

It is not surprising that tumors, as well as viruses, have evolved mechanisms to interfere with proteasome function. The extent of this interference is uncertain, but interference with molecules that are required for efficient antigen processing and presentation has been reported. Many tumors have down-regulated TAP1, TAP2, PSMB9 (LMP2) or PSMB8 (LMP7) expression (*31, 32*); re-establishing these pathways by gene transfer often leads to immune recognition of these tumors. One mechanism by which tumors may escape immune recognition is the release of high levels of the immunosuppressive cytokine interleukin 10, which inactivates TAP and proteasomal function, leading to decreased presentation of peptide-epitopes on MHC I molecules on the cell surface (*33*). Modulation of proteasome activity by cytokines may also enhance tumor antigen presentation. Studies in our laboratories showed that *IL3* gene transfection of fibrosarcoma cells enhanced proteasomal activity and the immunogenicity of immunogenic fibrosarcoma cells when compared to nonimmunogenic control cells expressing a control vector (Pajonk and McBride, unpublished data).

In addition to cancer and viral infections, there is growing evidence that the proteasome/TAP system plays a crucial immunomodulatory role in benign inflammatory diseases. Recent studies have linked polymorphism of 26S proteasome subunits and TAP to Sjoergren's syndrome, streptococcal-related polyarthritis, rheumatoid arthritis, myositis, systemic lupus erythematosus, HLA-B27-associated juvenile rheumatoid arthritis, and juvenile ankylosing spondylitis (*34–40*). The critical role of the proteasome in the generation of immunity is supported by recent studies identifying the frequently used immunosuppressive drugs cyclosporin A and rapamycin as potent and direct inhibitors of proteasome activity (*41, 42*).

PROTEASOMES AND CANCER

Tumor Suppressor and Oncogenes

It is widely accepted that most, if not all, forms of malignancy are caused by genetic mutations in oncogenes and/or tumor suppressor genes. It is of interest to note that proteasomal degradation regulates the level of expression of the products of many of these genes (Tables 1 and 2) (*43–45*), and any cancer-related modifications in proteasome function may affect the degradation pathways. Furthermore, some gene products interact directly, or indirectly through the ubiquitin pathway, with proteasomal subunits. Mutations in ubiquitin or proteasome binding sites could alter protein stability and contribute to carcinogenesis. In addition, some tumor suppressor gene or oncogene products actually participate in substrate ubiquitinylation as E3 ligases. The potential implications of these interactions can be seen by examination of a number of examples.

Mutations in the tumor suppressor gene *APC* have been associated with the development of familial polyposis coli and spontaneous colon carcinomas. One function of the APC gene product is the control of β-catenin levels. Mutation of the *APC* gene leads to accumulation of β-catenin in the cytosol, and increased β-catenin levels have been linked to human cancer (reviewed in ref. *46*). The mechanism by which APC controls β-catenin levels is not fully understood, but APC is involved in its ubiquitination (*47*),

TABLE 1
Human Tumor Suppressor Genes Interacting with the Ubiquitin-26S Proteasome Pathway

Gene	Tumor	Type of interaction	Reference(s)
APC	Familial adenomatosis polyposis	Blocks β-catenin degradation by the 26S proteasome	(*47*)
DCC	Colon carcinoma	Degraded by the 26S proteasome	(*52, 175*)
TP53	Breast, colon, lung carcinomas, osteosarcoma, astrocytoma, etc.	Degraded by the 26S proteasome	(*176–178*)
RB1	Retinoblastoma, osteosarcoma, breast, bladder, lung carcinomas	Degraded by the 26S proteasome	(*29*)
VHL	von Hippel-Lindau syndrome (renal carcinoma, pheochromocytoma, hemangioblastoma)	Targets HIF1A for 26S proteasome-dependent degradation under well-oxygenated conditions	(*66*)

and *APC* mutation may interfere with its phosphorylation (*48*).

The tumor suppressor gene *DCC* (deleted in colon cancer) spans a 1.4-Mbp region of the human genome (*49*), and deletions in this region are observed in about 70% of colorectal cancers (*50*). The functions of its gene products are not clear, but the main product is a large transmembrane protein of four immunoglobulin-like and six fibronectin type III-like extracellular domains with a 325-amino-acid cytoplasmic domain (*51*). The cytoplasmic domain has recently been shown by yeast two-hybrid screening to bind SIAH proteins, the human homologues of the *Drosophila* seven in absentia (sina) gene. SIAH regulates the stability of DCC by interacting with ubiquitinating enzymes that target DCC for degradation (*52*).

The *TP53* tumor suppressor gene is mutated in about half the cases of human cancer. It is expressed at low levels in normal cells, but levels are elevated after mutation or in response to DNA damage, such as that caused by ionizing radiation. The level of TP53 is determined by nontranscriptional mechanisms. TP53 is targeted for destruction by the E3 ubiquitin ligase MDM2, and disruption of this autoregulatory loop has profound effects on cell survival and tumorigenesis. Conjugation of UBL1 (also known as SUMO-1) with MDM2 prevents self-ubiquitination and degradation of MDM2, increasing the rate of destruction of TP53 (*53*). Stabilization of TP53 in response to radiation is associated with inhibition of MDM2-mediated degradation, reduction in MDM2 sumoylation, and phosphorylation of TP53 (*53*). Expression of TP53-induced CDKN1A (also known as $p21^{WAF1/Cip}$), which is responsible in large part for radiation-induced G_1/S-phase arrest through CDK inhibition, and GADD45, which is involved in G_2/M-phase arrest, is controlled directly and indirectly by proteasomal degradation. Stabilization of mutated TP53 seems to be the result of two independent phenomena, both of which cause its impaired ubiquitination: The loss of wild-type TP53 function acts to stabilize mutated TP53 by affecting MDM2-mediated ubiquitination. In addition, mutated TP53 can be stabilized by binding of HSP90, which could be overcome by treatment with the HSP90 inhibitor geldanamycin (*54*).

The protein product of the gene mutated in retinoblastoma (*RB1*, also known as RB, pRB or p105), is a negative regulator of the G_1- to S-phase transition. The hypophos-

TABLE 2
Human Proto-Oncogenes Interacting with the Ubiquitin-26S Proteasome Pathway

Gene/ gene family	Tumors with abnormal expression	Type of interaction	Reference(s)
ABL	Chronic myelocytic leukemia, acute lymphatic leukemia, chronic neutrophilic leukemia	BCR-ABL targets ABI for proteasomal degradation. BCR-ABL expression depends on 26S proteasome function	(*77, 78*)
FOS	Breast, ovarian, prostate, cervical cancer, skin	Degraded by the 26S proteasome	(*93, 94, 96*)
MOS	Plasmocytoma	Degraded by the 26S proteasome	(*179*)
MYB	Myeloid and lymphoid leukemia	Degraded by the 26S proteasome	(*99*)
MYC	B-cell lymphomas, promyelocytic leukemia	Degraded by the 26S proteasome	(*106*)
RAF		Raf-B binds to PA28α activates the 26S proteasome; RAF1 is degraded by the 26S proteasome	(*110, 180*)
RAS	Lung, colon, bladder, breast and teratocarcinoma, neuroblastoma, leukemia, fibrosarcoma, melanoma, rhabdomyosarcoma	Inhibits the proteasome-dependent degradation of *MYC*	(*44*)
REL	B-cell lymphomas, multiple myeloma, Hodgkin's disease, non-small cell lung carcinoma, squamous head and neck carcinoma, breast cancer, colon cancer, stomach cancer, thyroid carcinoma	Subunit of the transcription factor NFKB, activated by 26S proteasome-dependent degradation of NFKBI	(*114*)
SRC	Brain tumors	Targets ABI for proteasomal degradation	(*77*)

phorylated form of RB1 binds to the E2F family of transcription factors that controls expression of essential cell cycle proteins like DNA polymerase α. Upon phosphorylation by cyclin-dependent kinases (CDKs), RB1 releases E2F, allowing transcriptional activation of E2F-dependent genes and cell cycle progression (*55*). RB1 is ubiquitinated and degraded (*29, 56–59*). Additionally, free E2F itself is degraded in a ubiquitin-dependent manner by 26S proteasome [for a review of E2F regulation, see (*60*)]. Mutated RB1 that has been deleted in at the carboxy-terminal end (position 1–792) lacks E2F stabilizing function and is unable to protect E2F from ubiquitination and subsequent degradation by the 26S proteasome (*56*).

In response to low oxygen tension, cells activate the transcription factor HIF1, which binds to specific DNA sequences in the promoter regions of genes like erythropoietin and vascular endothelial growth factor (*VEGF*). HIF1 is a heterodimer of HIF1A and HIF-1β (now known as aryl hydrocarbon receptor nuclear translocator, ARNT). In addition to binding to HIF1A, ARNT is able to bind to the aryl hydrocarbon receptor (AHR) and subsequently activates genes of the xenobiotic detoxification system (*61*). Under well-oxygenated conditions, HIF1A is rapidly inactivated by 26S proteasome-dependent degradation (*62*). Under hypoxic conditions, HIF1A is stabilized and active (*63*). Hypoxia inhibits proteasome function (*64*), indicating one of the mechanisms by which this stabilization might occur. The von Hippel-Lindau tumor suppressor gene product (VHL), which is thought to be regulated by a ferro-protein oxygen sensor (*65*), is a component of the E3 ligase complex that leads to degradation of HIF1A (*66*). Two recent reports indicate that HIF1A is hydroxylated at a proline residue under well-oxygenated conditions and that this post-translational modification targets HIF1A for ubiquitination by VHL (*67–69*). Mutations in *VHL* are found in patients with dominant inherited VHL syndrome; this is the most common genetic defect associated with kidney cancer in humans. Such defects cause accumulation of HIF1A followed by uncontrolled angiogenesis as a result of HIF1 transcriptional activity. The data suggest a direct link between the VHL tumor suppressor gene product and the process of ubiquitination and its dysregulation in kidney cancer.

ABL is the cellular homologue of the transforming gene of Abelson murine leukemia virus. Activation of the oncogenic potential of *ABL* occurs as a consequence of a translocation event that results in the expression of chimeric fusion proteins like BCR-ABL and ETV6 (also known as TEL)-ABL (*70*) in chronic myelogenous leukemia (*70*), acute lymphocytic leukemia (*71*), and chronic neutrophilic leukemia (*72, 73*). ABL codes for a tyrosine kinase that carries 3 DNA-binding domains and is a negative regulator of cell growth (*74*). Intranuclear proteins such as RB1 negatively regulate ABL kinase activity, and proteasomal degradation of RB1 releases ABL to phosphorylate RNA polymerase II. After irradiation, ABL is phosphorylated by ATM (reviewed in ref. *75*). In cells carrying the BCR-ABL translocation, the fusion protein relocates from the nucleus to the cytoplasm and shows greatly enhanced tyrosine kinase activity, resulting in positive regulation of cell growth (*76*). Dai and coworkers recently reported that BCR-ABL targets ABL-interactor proteins (ABI1 and ABI2), which are inhibitors of the tyrosine kinase activity of ABL, for ubiquitin-dependent proteolysis by the 26S proteasome (*77*). The dependence of BCR-ABL on the 26S proteasome pathway for its growth-promoting function is further supported by the observation that proteasome inhibition leads to inactivation of BCR-ABL function, reduced BCR-ABL expression, and apoptosis in K562 cells (*78*). Mild hyperthermia, which also inhibits proteasome function, has a similar effect on BCR-ABL expression (*79*).

Abnormal expression levels of FOS have been found in breast cancer (*80*), ovarian cancer (*81*), prostate cancer (*82*), cervical cancer (*83*), and skin carcinomas (*84*). FOS, the gene product of the *FOS* proto-oncogene, is a major subunit of the activator protein 1 (AP1) transcription factor complex, a pathway involved in cell growth (*85*), differentiation (*86*) and transformation (*87*). AP1 activation results from signaling through MAP kinase pathways. Activation of the MAPK8 (also known as JNK/SAPK) pathway leads to up-regulated transcription of FOS and phosphorylation of JUN at serine 63 and 73 (*88*). This leads to formation and enhanced transcriptional potential of the AP1 transcription factor complex (*89–92*). These pathways are counterbalanced by ubiquitin-dependent proteolysis of FOS (*93–96*) and JUN (*94, 95, 97*) by the 26S proteasome. Although the conjugating enzymes responsible for ubiquitination of FOS have been identified (*96*), the pathways and signals that target FOS and JUN for ubiquitination are not fully understood, but deletion of the C-terminal PEST sequence, which is altered in the proto-oncogene *FOS*, greatly enhances its stability (*48*).

MYB is the human homologue of the avian gene that was first described in avian myeloid leukemia viruses, and it is associated with development of myeloid leukemia in humans. It is up-regulated during the G_1 phase of the cell cycle in hematopoietic cells. In these cells, MYB functions as a transcription factor involved in proliferation and differentiation (reviewed in ref. *98*). MYB proteins have a half-life of less than 1 h (*99, 100*). This is achieved by ubiquitin-dependent degradation by the 26S proteasome. Myeloid leukemia-specific forms of MYB that are truncated at the COOH-terminal exhibited increased stability (*99*).

The *MYC* gene family consists of cellular *MYC, MYCN*, which is associated with neuroblastomas (*101*) and retinoblastomas (*102*), and *MYCL*, which is detected in small cell lung cancers (*103, 104*). In the presence of RAS, MYC has transforming activity, and dysregulated levels of MYC have been described for many malignancies. MYC has a very short half-life of 30 min (*105*), and MYC and MYCN are both degraded by the 26S proteasome in a ubiquitin-dependent manner (*106*). This process is promoted by the human

papilloma virus protein E6, which is expressed by the highly oncogenic strain HPV16 (*106*).

Many extracellular survival signals converge in activation of the RAS/RAF1/MAP kinase pathway (reviewed in ref. *107*), leading to cell differentiation (*108*) or proliferation (*109*). RAF1 is normally complexed in a cell with CDC37 (p50), which recruits HSP90 to stabilize RAF1. RAF1 is rapidly degraded by the 26S proteasome in its absence (*110, 111*). Disruption of RAF1-HSP90 complex formation using the HSP90 inhibitor geldanamycin, a benzoquinone, or ansamycin or by overexpression of a dominant negative CDC37 (p50) that is unable to recruit HSP90 causes failure of signal transduction (*112*). RAF1 mutations have been described in human lymphomas and leukemias (*113*), but it is not known whether these mutations lead to the stabilization of RAF1 by the blocking of proteasome-dependent degradation.

The members of the REL/NFKB family of transcription factors share a highly conserved DNA-binding domain called the REL homology domain. Homo- or heterodimers of this family bind to 10-bp DNA sites (κ-sites). Mammals have five different NFKB subunits: p50 (TNFRSF5)/p105 (NFKB1), p52/p100 (NFKB2), REL, p65/RELA and RELB. The p65/p50 heterodimer is most important for most responses. It is sequestered in the cytoplasm bound to its inhibitor molecule NFKB1A. The classical pathway of NFKB activation involves phosphorylation of NFKB1A by IKK kinases, causing its ubiquitination and subsequent degradation by the 26S proteasome. This frees NFKB for translocation into the nucleus and allows transcriptional activation of NFKB-dependent genes (reviewed in ref. *114*).

Constitutive activation of the NFKB signal transduction pathway has been implicated as promoting cell survival in many malignancies, including non-Hodgkin's lymphoma, Hodgkin's disease (*115*), myeloma, breast cancer, prostate cancer (*116*), melanoma (*117*), and squamous cell carcinoma of the head and neck (*118*). Activation can be the result of gene amplification or rearrangements of the *REL* gene (lymphomas), *REL* overexpression (non-small cell lung carcinomas), *RELA* translocation, overexpression or amplification (lymphomas and leukemias, squamous cell carcinoma of the head and neck, adenocarcinoma of the breast and the stomach, and thyroid carcinomas), overexpression of TNFRF5 (non-small cell lung carcinomas), or mutations in NFKB1A (Hodgkin's disease) (reviewed in ref. *119*). One potential additional mechanism leading to constitutive NFKB activation is increased 26S proteasome activity, which could enhance the rate of degradation of NFKBI, or increase the production of TNFRSF5 from NFKB1. Proteasome activity and NFKB activity correlated well in a panel of human tumor cell lines (*120*).

The *SRC* oncogene encodes for the tyrosine kinase $pp60^{SRC}$, which was first described by Duesberg and Vogt (*121*). It is the prototype for a family of related kinases that are involved in many signal transduction pathways. Abnormal expression of this gene has been described in human brain tumors (*122*). The gene product $pp60^{SRC}$ interacts with ubiquitin/26S proteasome pathway in two ways: Oncogenic forms of $pp60^{SRC}$ phosphorylate ABI proteins and target them for ubiquitination and degradation (*77*). ABI proteins in turn antagonize the oncogenic potential of ABL, giving rise to a contribution of *SRC* to the progression of BCR-ABL-positive leukemias. Additionally, wild-type $pp60^{SRC}$ is itself degraded through the 26S proteasome (*123, 124*).

PROTEASOMES AND CELL FUNCTION

Cell Cycle Regulation

The eukaryotic cell cycle is coordinated by the interaction of families of cyclins with cyclin-dependent kinases (CDKs). Cyclin levels vary throughout the cell cycle, and their regulated degradation by the proteasome is essential for cell cycle progression. Degradation is facilitated by polyubiquitination by a family of E3 ubiquitin ligases termed the SKP1-CDC53-F-box protein (SCF) complex (*125*). Rapidly proliferating cells, whether they are progenitor cells or cancer cells, generally show increased levels of expression of proteasome subunits (*120, 126, 127*). Inhibition of proteasome function arrests cells in G_1 (*128–130*), late S (*131*), and G_2/M phase of the cell cycle (*132*).

The Role of the Proteasome in Catabolic States

Cancer is frequently associated with an increased rate of catabolism, as are several other pathological conditions, including chronic renal failure and sepsis. The increased catabolism is often linked to altered cytokine profiles, in particular increased levels of TNF and other proinflammatory cytokines, and/or to acidosis, and is characterized by progressive muscle protein loss (*133, 134*) and negative nitrogen balance (*135*). An inherent component of the imbalance appears to be increased proteasome activity. The cause of the increase is not known, but cytokines and acidosis may directly alter proteasome structure and function. This may explain why correction of low pH by bicarbonates corrects muscle protein loss in patients with chronic renal failure (*135, 136*).

The Proteasome in Angiogenesis and Erythropoiesis

Tumor growth requires the induction of new blood vessels to provide oxygen and nutrients. The process of angiogenesis depends on two critical steps: the sensing of low oxygen tensions and the subsequent transcriptional activation of pathways leading to production of growth factors that initiate angiogenesis and stimulate erythropoiesis. Both steps depend critically on proteasome function. As mentioned earlier, low oxygen tensions cause HIF1A to dimerize with ARNT. At present, it is not clear whether HIF1A must be activated in response to hypoxia or whether hypoxia-mediated inhibition of proteasome-dependent degradation is sufficient to regulate its activity, but there is strong evidence that the latter is important (*63*). Translocation of

HIF1A/ARNT to the nucleus causes transcription that includes the *VEGF* and erythropoietin (*EPO*) genes. The former acts on endothelial cells to initiate angiogenesis, while the latter promotes oxygen delivery. Signaling through the erythropoietin receptor (EPOR) and expression of the receptor itself are regulated by controlled proteolysis through the 26S proteasome (*137, 138*). Since angiogenesis within tumors through the HIF1A pathway is regulated by the ubiquitin/proteasome pathway, this pathway offers a promising target for future therapies directed at tumor-related anemia and tumor anti-angiogenesis.

The Proteasome and Apoptosis

One of the most striking observations in proteasome research is the fact that inhibitors of the proteasome induce apoptosis in almost every malignant cell line (*78, 120, 139–147*). Some normal cell types, such as thymocytes, are initially rescued by proteasome inhibitors from apoptosis induced by ionizing radiation, glucocorticoids or phorbol ester (*148*). Long-term inhibition of proteasomes will eventually result in cell death, but there appears to be a difference between normal and cancer cells that may be due to their proliferative status or to cancer cells relying more on stress pathways, both of which would be affected rapidly by proteasome inhibition.

Life without proteasome function is usually impossible, although compensatory mechanisms have been observed after extensive selection of EL-4 mouse lymphoma cells (*149*) and human PC-3 prostate carcinoma cells (Pajonk and McBride, unpublished results) that survived in the presence of a proteasome inhibitor. In EL-4 cells, TPPII, a giant protease, was shown to compensate for the loss of proteasome activity and to allow cells to escape cell death resulting from proteasome inhibition (*149, 150*).

The mechanism of apoptosis induced by proteasome inhibitors is incompletely understood and may vary with the cell line [for a recent review, see ref. (*151*)]. Drexler, as well as Soldatenkov and Dritschilo (*152*), suggested that activation of a BCL2-sensitive pathway is involved. In contrast, Hermann and coworkers excluded any involvement of BCL2 (*142*). We and others (*120, 142*) have demonstrated TP53-independent cell death using the MG-132 proteasome inhibitor. Caspase inhibitors were able to prevent DNA fragmentation but not apoptosis caused by lactacystin treatment of MO7e human myeloid progenitor cells, suggesting that caspase activation was a secondary effect rather than a direct effect (*141*). In our own studies, MG-132, which also inhibits calpain, did not cause caspase 3 activation in PC-3 prostate cancer cells (Pajonk and McBride, submitted for publication), which might be because calpain activity is necessary to cleave procaspase 3 to its active form (*153*). However, the cells still died by apoptosis.

Proteasome Function and Cancer Treatment

1. Proteasome function and radiation therapy

Studies in our laboratory recently demonstrated that proteasome inhibition radiosensitizes SiHa cervical cancer cells (Pajonk *et al.*, unpublished results), PC-3 prostate cancer cells (Pajonk and McBride, submitted for publication), and HD-MyZ Hodgkin's lymphoma cells (*120*). Comparable results have been reported for EMT-6 tumors (*154*). In our experiments, radiosensitization did not depend on the level of expression or activity of PRKDC (also known as DNA-PKcs) or on TP53 status. The mechanism of radiosensitization is unclear. One possibility is inhibition of the action of CDC25A, a phosphatase that is usually rapidly degraded by the 26S proteasome after exposure of cells to ionizing radiation and is required for the G_1- to S-phase transition of the cell cycle. Overexpression of CDC25A leads to enhanced DNA damage and decreased cell survival (*155*), but its role in radiosensitization induced by proteasome inhibitors has not been established. Recent observations also suggest that the proteasome is intimately involved in the control of DNA repair, although how this process is affected by proteasome inhibition is not known. In any event, these observations suggest that proteasome inhibitors might be an interesting new class of radiosensitizing drugs. The reversible proteasome inhibitor PS-341 has entered clinical trials as a single agent with some success, in particular in multiple myeloma and leukemia (J. Adams, personal communication). Toxicity does not seem to be a major problem, provided that the agent is given every four days to spare the gastrointestinal tract. The fact that a therapeutic differential can be obtained may be explained by proliferating or "stressed" cells being preferentially targeted.

Ionizing radiation itself can lead to rapid, dose-dependent inhibition of proteasome function to 60% of baseline levels (*156*). In ECV304 cells, this decrease occurred within the dose range of 0.2 to 2 Gy and was not increased further with higher doses up to 20 Gy. Inhibition could be achieved almost immediately after irradiation, and the effect lasted for up to 24 h. The implications of this finding are potentially important when considering radiation-induced protein expression. Expression of many proteins, such as TP53, JUN, FOS, TNF and NFKB, is rapidly up-regulated after irradiation by post-transcriptional mechanisms. Rapid inhibition of proteasome function provides a means by which this could be achieved.

The interplay between nontranscriptional and transcriptional control mechanisms in protein expression is of interest and could help provide an explanation for nonlinear dose–response curves. For example, the transcription factor NFKB, the major mediator of inflammatory responses, is activated by radiation. If proteasome function is inhibited by radiation, one would expect NFKB activation to be inhibited, not activated, because NFKBI degradation would be blocked. This apparent paradox can be resolved, because in many cells NFKB is activated only after high doses and in some situations may use a nonclassical pathway and does not involve a decrease in NFKBI expression (*157*). At lower doses, inhibition of NFKB activation has been detected, at least in one cell line (*156*). The universality of the effect has yet to be established, but if it is, it is possible that the clinically established daily fractions of 2 Gy were chosen in part because this was a dose that minimized inflammation, in addition to sparing late-responding normal tissues.

It is of interest that for treatment of benign inflammatory

and hyperproliferative diseases like insertion tendonitis (*158*) arthrosclerosis (*159*), vascular restenosis (*160*), arteriovenous malformations (*161*), endocrine ophthalmopathy (*162, 163*), pterygium (*162, 164*), induratio penis plastica (*165*), keloids and heterotopic ossifications (*161*), the doses of radiation that were used were often lower than those used for cancer treatment. The anti-inflammatory action of ionizing radiation has not been fully explained, but if the proteasome is inhibited at low doses, this could inhibit NFKB activation in this dose region. Also, given the observation that inhibition of proteasome function has a radiosensitizing effect on cancer cells, our findings could help to explain the hypersensitivity of mammalian cells to low-dose irradiation described by Joiner and coworkers (*166*) and others. Radiation-induced DNA damage might be amplified by the radiosensitizing effect of the proteasome inhibition caused by low-dose irradiation.

2. Proteasome function and chemotherapy

Proteasome inhibition is a promising way to induce cell death by apoptosis and to radiosensitize chemotherapy- and radiation-resistant cancers (*120, 143, 145–147*). Additionally, recent data indicate the possible direct involvement of the proteasome in the mechanism of action of chemotherapeutic agents and in resistance to chemotherapy: For example, the anthracycline antibiotic doxorubicin accumulates rapidly in the nucleus of malignant cells. Doxorubicin is known to bind to high-molecular-weight proteins that exhibit chymotrypsin-like proteolytic activity. It has been suggested that these are proteasome subunits and that, because many proteasome subunits contain nuclear translocation signals and proteasome subunit expression is increased in malignant cells, the proteasome acts as a carrier for nuclear uptake of doxorubicin (*167*).

Bleomycin hydrolase, which deamidates the anti-cancer drug bleomycin, is a neutral cysteine protease with structural similarity to the 20S proteasome. Yeast two-hybrid studies identified the human homologue of yeast ubiquitin-conjugating enzyme 9 (UBC9) as a binding partner for bleomycin hydrolase, linking the ubiquitin system to chemotherapy resistance (*168*). Also, preclinical studies have shown that the proteasome inhibitor PS-341 (*169*) has additive antitumor effects when combined with 5-fluorouracil, cisplatin, paclitaxel and Adriamycin (*154*). Furthermore, it is remarkable that resistance to chemotherapy based on expression of the P-glycoprotein (P-gp), coded by the multidrug resistance gene 1 (*ABCB1*, also known as *MDR1*), can be overcome by co-administration of substances like cyclosporin A (*170*), and HIV-1 protease inhibitors like ritonavir (*171*), MG-132 (*172*) or PS-341 (*173*), which share an inhibitory effect on proteasome function. In the presence of MG-132, ubiquitinated forms of P-glycoprotein accumulate, but since lactacystin failed to inhibit P-gp function, the exact functional interaction with the proteasome pathway is not clear (*172*). As with radiation therapy, the utility of proteasome inhibitors with chemotherapeutic agents will depend on the therapeutic benefit that can be obtained. The first clinical trials using the proteasome inhibitor PS-341 seem promising, because this drug is surprisingly well tolerated when applied systemically (J. Adams, personal communication), although the basis for any differential effect remains elusive, other than perhaps a tendency to target cycling cells (*174*).

CONCLUDING REMARKS

Our understanding of how protein expression is regulated within a cell has improved dramatically in recent years. While most attention has been focused on the pathways of gene transcription, it is now well accepted that post-transcriptional mechanisms are also important. The role of the proteasome pathway as a post-translational control mechanism has been extended so that it now appears to leave almost no area of biological research untouched. Once thought to contain little specificity, the proteolytic process mediated by the proteasome now is seen as an exceptionally well-regulated pathway with high specificity, most of which lies in the family of E3 ubiquitin ligases, many of which have yet to be identified. In the future, targeting these E3 enzymes using competitive inhibitors might be an efficient and highly specific pharmacological way to manipulate almost any pathway involved in the pathophysiology of cancer.

Other levels of control over proteolysis also operate, and it is clear that proteasome function can be modified by cytokines, ionizing radiation, heat, hypoxia/reperfusion, and other oxidative stresses. The proteasome itself is therefore a highly responsive system that functions in concert with phosphorylation and dephosphorylation to allow cells to make rapid and appropriate initial responses to a wide variety of insults. Its role in carcinogenesis and cancer treatment is only beginning to be understood, but it is going to be a prime topic for proteomic research in the immediate future.

REFERENCES

1. K. Tanaka, T. Tamura, N. Tanahashi and C. Tsurumi. Protein and gene structures of 20S and 26S proteasomes. *Adv. Exp. Med. Biol.* **389**, 187–95 (1996).
2. W. Dubiel, K. Ferrell and M. Rechsteiner, Subunits of the regulatory complex of the 26S protease. *Mol. Biol. Rep.* **21**, 27–34 (1995).
3. A. J. Koster, J. Walz, A. Lupas and W. Baumeister, Structural features of archaebacterial and eukaryotic proteasomes. *Mol. Biol. Rep.* **21**, 11–20 (1995).
4. T. Tamura, I. Nagy, A. Lupas, F. Lottspeich, Z. Cejka, G. Schoofs, K. Tanaka, R. De Mot and W. Baumeister, The first characterization of a eubacterial proteasome: The 20S complex of Rhodococcus. *Curr. Biol.* **5**, 766–774 (1995).
5. W. Hilt and D. H. Wolf, Proteasomes of the yeast *S. cerevisiae*: Genes, structure and functions. *Mol. Biol. Rep.* **21**, 3–10 (1995).
6. N. Tanahashi, C. Tsurumi, T. Tamura and K. Tanaka, Molecular structure of 20S and 26S proteasomes. *Enzyme Protein* **47**, 241–251 (1993).
7. A. Ciechanover, The ubiquitin-proteasome proteolytic pathway. *Cell* **79**, 13–21 (1994).
8. D. H. Lee and A. L. Goldberg, Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. *J. Biol. Chem.* **271**, 27280–27284 (1996).
9. C. Wojcik, K. Tanaka, N. Paweletz, U. Naab and S. Wilk, Proteasome activator (PA28) subunits, alpha, beta and gamma (Ki antigen) in

NT2 neuronal precursor cells and HeLa S3 cells. *Eur. J. Cell. Biol.* **77**, 151–160 (1998).

10. K. Tanaka, Molecular biology of proteasomes. *Mol. Biol. Rep.* **21**, 21–26 (1995).
11. R. Stohwasser and P. M. Kloetzel, Cytokine induced changes in proteasome subunit composition are concentration dependent. *Biol. Chem.* **377**, 571–577 (1996).
12. D. Nandi, E. Woodward, D. B. Ginsburg and J. J. Monaco, Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor beta subunits. *EMBO J.* **16**, 5363–5375 (1997).
13. M. Schmidt and P. M. Kloetzel, Biogenesis of eukaryotic 20S proteasomes: the complex maturation pathway of a complex enzyme. *FASEB J.* **11**, 1235–1243 (1997).
14. P. M. Kloetzel, A. Soza and R. Stohwasser, The role of the proteasome system and the proteasome activator PA28 complex in the cellular immune response. *Biol. Chem.* **380**, 293–297 (1999).
15. M. P. Belich and J. Trowsdale, Proteasome and class I antigen processing and presentation. *Mol. Biol. Rep.* **21**, 53–56 (1995).
16. M. Groettrup, A. Soza, U. Kuckelkorn and P. M. Kloetzel, Peptide antigen production by the proteasome: Complexity provides efficiency. *Immunol. Today* **17**, 429–435 (1996).
17. N. Shimbara, K. Ogawa, Y. Hidaka, H. Nakajima, N. Yamasaki, S. Niwa, N. Tanahashi and K. Tanaka, Contribution of proline residue for efficient production of MHC class I ligands by proteasomes. *J. Biol. Chem.* **273**, 23062–23071 (1998).
18. C. A. Realini and M. C. Rechsteiner, Proposed role of a gamma-interferon inducible proteasome-regulator in antigen presentation. *Adv. Exp. Med. Biol.* **389**, 51–61 (1996).
19. C. Sibille, K. G. Gould, K. Willard-Gallo, S. Thomson, A. J. Rivett, S. Powis, G. W. Butcher and P. De Baetselier, LMP2+ proteasomes are required for the presentation of specific antigens to cytotoxic T lymphocytes. *Curr. Biol.* **5**, 923–930 (1995).
20. H. J. Fehling, W. Swat, C. Laplace, R. Kühn, K. Rajewsky, U. Müller and H. von Boehmer, MHC class I expression in mice lacking the proteasome subunit LMP-7. *Science* **265**, 1234–1237 (1994).
21. L. Van Kaer, P. G. Ashton-Rickardt, M. Eichelberger, M. Gaczynska, K. Nagashima, K. L. Rock, A. L. Goldberg, P. C. Doherty and S. Tonegawa, Altered peptidase and viral-specific T cell response in LMP2 mutant mice. *Immunity* **1**, 533–541 (1994).
22. V. Cerundolo, A. Kelly, T. Elliott, J. Trowsdale and A. Townsend, Genes encoded in the major histocompatibility complex affecting the generation of peptides for TAP transport *Eur. J. Immunol.* **25**, 554–562 (1995); Erratum, *Eur. J. Immunol.* **25**, 1485 (1995).
23. K. Ferrell, C. R. Wilkinson, W. Dubiel and C. Gordon, Regulatory subunit interactions of the 26S proteasome, a complex problem. *Trends Biochem. Sci.* **25**, 83–88 (2000).
24. A. Hill, P. Jugovic, I. York, G. Russ, J. Bennink, J. Yewdell, H. Ploegh and D. Johnson, Herpes simplex virus turns off the TAP to evade host immunity. *Nature* **375**, 411–415 (1995).
25. R. Ehrlich, Modulation of antigen processing and presentation by persistent virus infections and in tumors. *Human Immunol.* **54**, 104–116 (1997).
26. E. Wiertz, A. Hill, D. Tortorella and H. Ploegh, Cytomegaloviruses use multiple mechanisms to elude the host immune response. *Immunol. Lett.* **57**, 213–216 (1997).
27. E. Berezutskaya and S. Bagchi, The human papillomavirus E7 oncoprotein functionally interacts with the S4 subunit of the 26 S proteasome. *J. Biol. Chem.* **272**, 30135–30140 (1997).
28. F. Mantovani and L. Banks, Inhibition of E6 induced degradation of p53 is not sufficient for stabilization of p53 protein in cervical tumour derived cell lines. *Oncogene* **18**, 3309–3315 (1999).
29. S. N. Boyer, D. E. Wazer and V. Band, E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res.* **56**, 4620–4624 (1996).
30. S. Tsubuki, Y. Saito and S. Kawashima, Purification and characterization of an endogenous inhibitor specific to the Z-Leu-Leu-Leu-MCA degrading activity in proteasome and its identification as heat-shock protein 90. *FEBS Lett.* **344**, 229–233 (1994).
31. A. Johnsen, J. France, M. S. Sy and C. V. Harding, Down-regulation of the transporter for antigen presentation, proteasome subunits, and class I major histocompatibility complex in tumor cell lines. *Cancer Res.* **58**, 3660–3667 (1998).
32. B. Seliger, M. J. Maeurer and S. Ferrone, TAP off—tumors on. *Immunol. Today* **18**, 292–299 (1997).
33. M. Petersson, J. Charo, F. Salazar-Onfray, G. Noffz, M. Mohaupt, Z. Qin, G. Klein, T. Blankenstein and R. Kiessling, Constitutive IL-10 production accounts for the high NK sensitivity, low MHC class I expression, and poor transporter associated with antigen processing (TAP)-1/2 function in the prototype NK target YAC-1. *J. Immunol.* **161**, 2099–2105 (1998).
34. E. Feist, U. Kuckelkorn, T. Dörner, H. Dönitz, S. Scheffler, F. Hiepe, P. M. Kloetzel and G. R. Burmester, Autoantibodies in primary Sjögren's syndrome are directed against proteasomal subunits of the alpha and beta type. *Arthritis Rheum.* **42**, 697–702 (1999).
35. V. J. Palombella, E. M. Conner, J. W. Fuseler, A. Destree, J. M. Davis, F. S. Laroux, R. E. Wolf, J. Huang, S. Brand and M. B. Grisham, Role of the proteasome and NF-κB in streptococcal cell wall-induced polyarthritis. *Proc. Natl. Acad. Sci. USA* **95**, 15671–15676 (1998).
36. J. Vinasco, A. Fraile, A. Nieto, Y. Beraun, E. Pareja, L. Mataran and J. Martín, Analysis of LMP and TAP polymorphisms by polymerase chain reaction-restriction fragment length polymorphism in rheumatoid arthritis. *Ann. Rheum. Dis.* **57**, 33–37 (1998).
37. E. Feist, T. Dörner, U. Kuckelkorn, G. Schmidtke, B. Micheel, F. Hiepe, G. R. Burmester and P. M. Kloetzel, Proteasome alpha-type subunit C9 is a primary target of autoantibodies in sera of patients with myositis and systemic lupus erythematosus. *J. Exp. Med.* **184**, 1313–1318 (1996).
38. K. G. Pryhuber, K. J. Murray, P. Donnelly, M. H. Passo, W. P. Maksymowych, D. N. Glass, E. H. Giannini and R. A. Colbert, Polymorphism in the LMP2 gene influences disease susceptibility and severity in HLA-B27 associated juvenile rheumatoid arthritis. *J. Rheumatol.* **23**, 747–752 (1996).
39. R. Ploski, B. Flatø, O. Vinje, W. Maksymowych, O. Forre and E. Thorsby, Association to HLA-DRB1*08, HLA-DPB1*0301 and homozygosity for an HLA-linked proteasome gene in juvenile ankylosing spondylitis. *Human Immunol.* **44**, 88–96 (1995).
40. W. P. Maksymowych and A. S. Russell, Polymorphism in the LMP2 gene influences the relative risk for acute anterior uveitis in unselected patients with ankylosing spondylitis. *Clin. Invest. Med.* **18**, 42–46 (1995).
41. S. Meyer, N. G. Kohler and A. Joly, Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-κB activation. *FEBS Lett.* **413**, 354–358 (1997).
42. X. Wang, S. Omura, L. I. Szweda, Y. Yang, J. Bérard, J. Seminaro and J. Wu, Rapamycin inhibits proteasome activator expression and proteasome activity. *Eur. J. Immunol.* **27**, 2781–2786 (1997).
43. H. Oda, S. Kumar and P. M. Howley, Regulation of the Src family tyrosine kinase Blk through E6AP-mediated ubiquitination. *Proc. Natl. Acad. Sci. USA* **96**, 9557–9562 (1999).
44. R. Sears, G. Leone, J. DeGregori and J. R. Nevins, Ras enhances Myc protein stability. *Mol. Cell* **3**, 169–179 (1999).
45. M. Fanelli, S. Minucci, V. Gelmetti, C. Nervi, C. Gambacorti-Passerini and P. G. Pelicci, Constitutive degradation of PML/RARα through the proteasome pathway mediates retinoic acid resistance. *Blood* **93**, 1477–1481 (1999).
46. L. C. Bullions and A. J. Levine, The role of beta-catenin in cell adhesion, signal transduction, and cancer. *Curr. Opin. Oncol.* **10**, 81–87 (1998).
47. V. Easwaran, V. Song, P. Polakis and S. Byers, The ubiquitin-proteasome pathway and serine kinase activity modulate adenomatous polyposis coli protein-mediated regulation of beta-catenin-lymphocyte

enhancer-binding factor signaling. *J. Biol. Chem.* **274**, 16641–16645 (1999).

48. S. Taya, T. Yamamoto, M. Kanai-Azuma, S. A. Wood and K. Kaibuchi, The deubiquitinating enzyme Fam interacts with and stabilizes beta-catenin. *Genes Cells* **4**, 757–767 (1999).
49. K. R. Cho, J. D. Oliner, J. W. Simons, L. Hedrick, E. R. Fearon, A. C. Preisinger, P. Hedge, G. A. Silverman and B. Vogelstein, The DCC gene: Structural analysis and mutations in colorectal carcinomas. *Genomics* **19**, 525–531 (1994).
50. E. R. Fearon, K. R. Cho, J. M. Nigro, S. E. Kern, J. W. Simons, J. M. Ruppert, S. R. Hamilton, A. C. Preisinger, G. Thomas and K. W. Kinzler, Identification of a chromosome 18q gene that is altered in colorectal cancers. *Science* **247**, 49–56 (1990).
51. L. Hedrick, K. R. Cho, E. R. Fearon, T. C. Wu, K. W. Kinzler and B. Vogelstein, The DCC gene product in cellular differentiation and colorectal tumorigenesis. *Genes Dev.* **8**, 1174–1183 (1994).
52. G. Hu, S. Zhang, M. Vidal, J. L. Baer, T. Xu and E. R. Fearon, Mammalian homologs of seven in absentia regulate DCC via the ubiquitin-proteasome pathway. *Genes Dev.* **11**, 2701–2714 (1997).
53. T. Buschmann, S. Y. Fuchs, C. G. Lee, Z. Q. Pan and Z. Ronai, SUMO-1 modification of Mdm2 prevents its self-ubiquitination and increases Mdm2 ability to ubiquitinate p53. *Cell* **101**, 753–762 (2000).
54. Y. Nagata, T. Anan, T. Yoshida, T. Mizukami, Y. Taya, T. Fujiwara, H. Kato, H. Saya and M. Nakao, The stabilization mechanism of mutant-type p53 by impaired ubiquitination: the loss of wild-type p53 function and the hsp90 association. *Oncogene* **18**, 6037–6049 (1999).
55. D. G. Johnson and R. Schneider-Broussard, Role of E2F in cell cycle control and cancer. *Front. Biosci.* **3**, d447–448 (1998).
56. M. R. Campanero and E. K. Flemington, Regulation of E2F through ubiquitin-proteasome-dependent degradation: stabilization by the pRB tumor suppressor protein. *Proc. Natl. Acad. Sci. USA* **4**, 2221–2226 (1997).
57. F. Hofmann, F. Martelli, D. M. Livingston and Z. Wang, The retinoblastoma gene product protects E2F-1 from degradation by the ubiquitin-proteasome pathway. *Genes Dev.* **10**, 2949–2959 (1996).
58. G. Hateboer, R. M. Kerkhoven, A. Shvarts, R. Bernards and R. L. Beijersbergen, Degradation of E2F by the ubiquitin-proteasome pathway: Regulation by retinoblastoma family proteins and adenovirus transforming proteins. *Genes Dev.* **10**, 2960–2970 (1996).
59. E. J. Smith, G. Leone and J. R. Nevins, Distinct mechanisms control the accumulation of the Rb-related p107 and p130 proteins during cell growth. *Cell Growth Differ.* **9**, 297–303 (1998).
60. N. Dyson, The regulation of E2F by pRB-family proteins. *Genes Dev.* **12**, 2245–2262 (1998).
61. S. Salceda, I. Beck and J. Caro, Absolute requirement of aryl hydrocarbon receptor nuclear translocator protein for gene activation by hypoxia. *Arch. Biochem. Biophys.* **334**, 389–394 (1996).
62. S. Salceda and J. Caro, Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.* **272**, 22642–22647 (1997).
63. L. E. Huang, J. Gu, M. Schau and H. F. Bunn, Regulation of hypoxia-inducible factor 1α is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **5**, 7987–7992 (1998).
64. T. Kamikubo and T. Hayashi, Changes in proteasome activity following transient ischemia. *Neurochem. Int.* **28**, 209–212 (1996).
65. E. R. Maher and W. G. Kaelin, Jr., von Hippel-Lindau disease. *Medicine* **76**, 381–391 (1997).
66. P. H. Maxwell, M. S. Wiesener, G. W. Chang, S. C. Clifford, E. C. Vaux, M. E. Cockman, C. C. Wykoff, C. W. Pugh, E. R. Maher and P. J. Ratcliffe, The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* **399**, 271–275 (1999).
67. M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J. M. Asara, W. S. Lane and W. G. Kaelin, Jr., HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing. *Science* **292**, 464–468 (2001).
68. P. Jaakkola, D. R. Mole, Y. M. Tian, M. I. Wilson, J. Gielbert, S. J. Gaskell, A. Kriegsheim, H. F. Hebestreit, M. Mukherji and P. J. Ratcliffe, Targeting of HIF-α to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. *Science* **292**, 468–472 (2001).
69. H. Zhu and H. F. Bunn, Signal transduction. How do cells sense oxygen? *Science* **292**, 449–451 (2001).
70. A. Gotoh and H. E. Broxmeyer, The function of BCR/ABL and related proto-oncogenes. *Curr. Opin. Hematol.* **4**, 3–11 (1997).
71. R. Kurzrock, M. Shtalrid, P. Romero, W. S. Kloetzer, M. Talpas, J. M. Trujillo, M. Blick, M. Beran and J. U. Gutterman, A novel c-abl protein product in Philadelphia-positive acute lymphoblastic leukaemia. *Nature* **325**, 631–635 (1987).
72. H. Wada, S. Mizutani, J. Nishimura, Y. Usuki, M. Kohsaki, M. Komai, H. Kaneko, S. Sakamoto, D. Delia and A. Kanamaru, Establishment and molecular characterization of a novel leukemic cell line with Philadelphia chromosome expressing p230 BCR/ABL fusion protein. *Cancer Res.* **55**, 3192–3196 (1995).
73. J. V. Melo, The diversity of BCR-ABL fusion proteins and their relationship to leukemia phenotype. *Blood* **88**, 2375–2384 (1996).
74. C. L. Sawyers, J. McLaughlin, A. Goga, M. Havlik and O. Witte, The nuclear tyrosine kinase c-Abl negatively regulates cell growth. *Cell* **77**, 121–131 (1994).
75. R. A. Van Etten, Cycling, stressed-out and nervous: Cellular functions of c-Abl. *Trends Cell Biol.* **9**, 179–186 (1999).
76. C. L. Sawyers, The bcr-abl gene in chronic myelogenous leukaemia. *Cancer Surv.* **15**, 37–51 (1992).
77. Z. Dai, R. C. Quackenbush, K. D. Courtney, M. Grove, D. Cortez, G. W. Reuther and A. M. Pendergast, Oncogenic Abl and Src tyrosine kinases elicit the ubiquitin-dependent degradation of target proteins through a Ras-independent pathway. *Genes Dev.* **12**, 1415–1424 (1998).
78. Q. P. Dou, T. F. McGuire, Y. Peng and B. An, Proteasome inhibition leads to significant reduction of Bcr-Abl expression and subsequent induction of apoptosis in K562 human chronic myelogenous leukemia cells. *J. Pharmacol. Exp. Ther.* **289**, 781–790 (1999).
79. S. K. Jain, I. de Aos, Y. Inai, F. Liu and L. Varticovski, Inactivation of wild-type BCR/ABL tyrosine kinase in hematopoietic cells by mild hyperthermia. *Leukemia* **14**, 845–852 (2000).
80. J. M. Gee, P. C. Willsher, F. S. Kenny, J. F. Robertson, S. E. Pinder, I. O. Ellis and R. I. Nicholson, Endocrine response and resistance in breast cancer: A role for the transcription factor Fos. *Int. J. Cancer* **84**, 54–61 (1999).
81. K. Luthra and T. N. Chapekar, Oncogene expression as detected by immunocytochemical staining in hormonally induced ovarian cell lines. *Indian J. Exp. Biol.* **36**, 447–455 (1998).
82. K. Aoyagi, I. Shima, M. Wang, Y. Hu, F. U. Garcia and M. E. Stearns, Specific transcription factors prognostic for prostate cancer progression. *Clin. Cancer Res.* **4**, 2153–2160 (1998).
83. T. H. Cheung, J. O. Leung, T. K. Chung, S. K. Lam, K. F. To and Y. F. Wong, c-fos overexpression is associated with the pathoneogenesis of invasive cervical cancer. *Gynecol. Obstet. Invest.* **43**, 200–203 (1997).
84. E. Saez, S. E. Rutberg, E. Mueller, H. Oppenheim, J. Smoluk, S. H. Yuspa and B. M. Spiegelman, c-fos is required for malignant progression of skin tumors. *Cell* **82**, 721–732 (1995).
85. F. Bost, R. McKay, N. Dean and D. Mercola, The JUN kinase/stress-activated protein kinase pathway is required for epidermal growth factor stimulation of growth of human A549 lung carcinoma cells. *J. Biol. Chem.* **272**, 33422–33429 (1997).
86. D. C. Kang, M. Motwani and P. B. Fisher, Role of the transcription factor AP-1 in melanoma differentiation. *Int. J. Oncol.* **13**, 1117–1126 (1998).
87. T. J. Bos, F. S. Monteclaro, F. Mitsunobu, A. R. Ball, Jr., C. H. Chang, T. Nishimura and P. K. Vogt, Efficient transformation of chicken em-

bryo fibroblasts by c-Jun requires structural modification in coding and noncoding sequences. *Genes Dev.* **4**, 1677–1687 (1990).

88. T. Hunter, Oncoprotein networks. *Cell* **88**, 333–46 (1997).
89. T. Smeal, B. Binetruy, D. A. Mercola, M. Birrer and M. Karin, Oncogenic and transcriptional cooperation with Ha-Ras requires phosphorylation of c-Jun on serines 63 and 73. *Nature* **354**, 494–496 (1991).
90. T. Smeal, B. Binetruy, D. Mercola, A. Grover-Bardwick, G. Heidecker, U. R. Rapp and M. Karin, Oncoprotein-mediated signalling cascade stimulates c-Jun activity by phosphorylation of serines 63 and 73. *Mol. Cell. Biol.* **12**, 3507–3513 (1992).
91. B. Binétruy, T. Smeal and M. Karin, Ha-Ras augments c-Jun activity and stimulates phosphorylation of its activation domain. *Nature* **351**, 122–127 (1991).
92. O. Potapova, A. Haghighi, F. Bost, C. Liu, M. J. Birrer, R. Gjerset and D. Mercola, The Jun kinase/stress-activated protein kinase pathway functions to regulate DNA repair and inhibition of the pathway sensitizes tumor cells to cisplatin. *J. Biol. Chem.* **272**, 14041–14044 (1997).
93. H. He, X. M. Qi, J. Grossmann and C. W. Distelhorst, c-Fos degradation by the proteasome. An early, Bcl-2-regulated step in apoptosis. *J. Biol. Chem.* **273**, 25015–25019 (1998).
94. C. Salvat, I. Jariel-Encontre, C. Acquaviva, S. Omura and M. Piechaczyk, Differential directing of c-Fos and c-Jun proteins to the proteasome in serum-stimulated mouse embryo fibroblasts. *Oncogene* **17**, 327–337 (1998).
95. M. L. Hermida-Matsumoto, P. B. Chock, T. Curran and D. C. Yang, Ubiquitinylation of transcription factors c-Jun and c-Fos using reconstituted ubiquitinylating enzymes. *J. Biol. Chem.* **271**, 4930–4936 (1996).
96. I. Stancovski, H. Gonen, A. Orian, A. L. Schwartz and A. Ciechanover, Degradation of the proto-oncogene product c-Fos by the ubiquitin proteolytic system *in vivo* and *in vitro*: Identification and characterization of the conjugating enzymes. *Mol. Cell. Biol.* **15**, 7106–7116 (1995).
97. A. M. Musti, M. Treier and D. Bohmann, Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* **275**, 400–402 (1997).
98. M. Introna, M. Luchetti, M. Castellano, M. Arsura and J. Golay, The myb oncogene family of transcription factors: Potent regulators of hematopoietic cell proliferation and differentiation. *Semin. Cancer Biol.* **5**, 113–124 (1994).
99. J. Bies and L. Wolff, Oncogenic activation of c-Myb by carboxyl-terminal truncation leads to decreased proteolysis by the ubiquitin-26S proteasome pathway. *Oncogene* **14**, 203–212 (1997).
100. S. Rogers, R. Wells and M. Rechsteiner, Amino acid sequences common to rapidly degraded proteins: The PEST hypothesis. *Science* **234**, 364–368 (1986).
101. N. E. Kohl, N. Kanda, R. R. Schreck, G. Bruns, S. A. Latt, F. Gilbert and F. W. Alt, Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell* **35**, 359–367 (1983).
102. W. H. Lee, A. L. Murphree and W. F. Benedict, Expression and amplification of the N-myc gene in primary retinoblastoma. *Nature* **309**, 458–460 (1984).
103. M. M. Nau, B. J. Brooks, J. Battey, E. Sausville, A. F. Gazdar, I. R. Kirsch, O. W. McBride, V. Bertness, G. F. Hollis and J. D. Minna, L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. *Nature* **318**, 69–73 (1985).
104. M. M. Nau, B. J. Brooks, Jr., D. N. Carney, A. F. Gazdar, J. F. Battey, E. A. Sausville and J. D. Minna, Human small-cell lung cancers show amplification and expression of the N-myc gene. *Proc. Natl. Acad. Sci. USA* **83**, 1092–1096 (1986).
105. G. Ramsay, L. Stanton, M. Schwab and J. M. Bishop, Human proto-oncogene N-myc encodes nuclear proteins that bind DNA. *Mol. Cell. Biol.* **6**, 4450–4457 (1986).
106. S. Gross-Mesilaty, E. Reinstein, B. Bercovich, K. E. Tobias, A. L. Schwartz, C. Kahana and A. Ciechanover, Basal and human papillomavirus E6 oncoprotein-induced degradation of Myc proteins by the ubiquitin pathway. *Proc. Natl. Acad. Sci. USA* **95**, 8058–8063 (1998).
107. D. K. Morrison and R. E. Cutler, The complexity of Raf-1 regulation. *Curr. Opin. Cell Biol.* **9**, 174–179 (1997).
108. K. W. Wood, H. Qi, G. D'Arcangelo, R. C. Armstrong, T. M. Roberts and S. Halegoua, The cytoplasmic raf oncogene induces a neuronal phenotype in PC12 cells: A potential role for cellular raf kinases in neuronal growth factor signal transduction. *Proc. Natl. Acad. Sci. USA* **90**, 5016–5020 (1993).
109. W. Kolch, G. Heidecker, P. Lloyd and U. R. Rapp, Raf-1 protein kinase is required for growth of induced NIH/3T3 cells. *Nature* **349**, 426–428 (1991).
110. T. W. Schulte, W. G. An and L. M. Neckers, Geldanamycin-induced destabilization of Raf-1 involves the proteasome. *Biochem. Biophys. Res. Commun.* **239**, 655–659 (1997).
111. L. F. Stancato, A. M. Silverstein, J. K. Owens-Grillo, Y. H. Chow, R. Jove and W. B. Pratt, The hsp90-binding antibiotic geldanamycin decreases Raf levels and epidermal growth factor signaling without disrupting formation of signaling complexes or reducing the specific enzymatic activity of Raf kinase. *J. Biol. Chem.* **272**, 4013–4020 (1997).
112. N. Grammatikakis, J. H. Lin, A. Grammatikakis, P. N. Tsichlis and B. H. Cochran, p50(cdc37) acting in concert with Hsp90 is required for Raf-1 function. *Mol. Cell. Biol.* **19**, 1661–1672 (1999).
113. G. C. Trench, M. Southall, P. Smith and C. Kidson, Allelic variation of the c-raf-1 proto-oncogene in human lymphoma and leukemia. *Oncogene* **4**, 507–510 (1989).
114. P. A. Baeuerle and D. Baltimore, NF-κB: Ten years after. *Cell* **87**, 13–20 (1996).
115. R. C. Bargou, C. Leng, D. Krappmann, F. Emmerich, M. Y. Mapara, K. Bommert, H. D. Royer, C. Scheidereit and B. Dörken, High-level nuclear NF-κB and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. *Blood* **87**, 4340–4347 (1996).
116. S. T. Palayoor, M. Y. Youmell, S. K. Calderwood, C. N. Coleman and B. D. Price, Constitutive activation of IκB kinase α and NF-κB in prostate cancer cells is inhibited by ibuprofen. *Oncogene* **18**, 7389–7394 (1999).
117. M. N. Devalaraja, D. Z. Wang, D. W. Ballard and A. Richmond, Elevated constitutive IκB kinase activity and IκB-α phosphorylation in Hs294T melanoma cells lead to increased basal MGSA/GRO-α transcription. *Cancer Res.* **59**, 1372–1377 (1999).
118. D. C. Duffey, Z. Chen, G. Dong, F. G. Ondrey, J. S. Wolf, K. Brown, U. Siebenlist and C. Van Waes, Expression of a dominant-negative mutant inhibitor-κα of nuclear factor-κB in human head and neck squamous cell carcinoma inhibits survival, proinflammatory cytokine expression, and tumor growth *in vivo*. *Cancer Res.* **59**, 3468–3474 (1999).
119. B. Rayet and C. Gelinas, Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **18**, 6938–6947 (1999).
120. F. Pajonk, K. Pajonk and W. McBride, Apoptosis and radiosensitization of Hodgkin's cells by proteasome inhibition. *Int. J. Radiat. Oncol. Biol.* **47**, 1025–1032 (2000).
121. P. H. Duesberg and P. K. Vogt, Differences between the ribonucleic acids of transforming and nontransforming avian tumor viruses. *Proc. Natl. Acad. Sci. USA* **67**, 1673–1680 (1970).
122. N. Takenaka, K. Mikoshiba, K. Takamatsu, Y. Tsukada, M. Ohtani and S. Toya, Immunohistochemical detection of the gene product of Rous sarcoma virus in human brain tumors. *Brain Res.* **337**, 201–207 (1985).
123. Y. Hakak and G. S. Martin, Ubiquitin-dependent degradation of active Src. *Curr. Biol.* **9**, 1039–1042 (1999).
124. K. F. Harris, I. Shoji, E. M. Cooper, S. Kumar, H. Oda and P. M. Howley, Ubiquitin-mediated degradation of active Src tyrosine kinase. *Proc. Natl. Acad. Sci. USA* **6**, 13738–13743 (1999).
125. A. R. Willems, T. Goh, L. Taylor, I. Chernushevich, A. Shevchenko and M. Tyers, SCF ubiquitin protein ligases and phosphorylation-dependent proteolysis. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **354**, 1533–1550 (1999).

126. A. Kumatori, K. Tanaka, N. Inamura, S. Sone, T. Ogura, T. Matsumoto, T. Tachikawa, S. Shin and A. Ichihara, Abnormally high expression of proteasomes in human leukemic cells. *Proc. Natl. Acad. Sci. USA* **87**, 7071–7075 (1990).

127. H. Kanayama, K. Tanaka, M. Aki, S. Kagawa, H. Miyaji, M. Satoh, F. Okada, S. Sato, N. Shimbara and A. Ichihara, Changes in expressions of proteasome and ubiquitin genes in human renal cancer cells. *Cancer Res.* **51**, 6677–6685 (1991).

128. S. I. Kumeda, A. Deguchi, M. Toi, S. Omura and K. Umezawa, Induction of G_1 arrest and selective growth inhibition by lactacystin in human umbilical vein endothelial cells. *Anticancer Res.* **19**, 3961–3968 (1999).

129. S. Hashemolhosseini, Y. Nagamine, S. J. Morley, S. Desrivieres, L. Mercep and S. Ferrari, Rapamycin inhibition of the G_1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. *J. Biol. Chem.* **273**, 14424–14429 (1998).

130. S. Rao, D. C. Porter, X. Chen, T. Herliczek, M. Lowe and K. Keyomarsi, Lovastatin-mediated G_1 arrest is through inhibition of the proteasome, independent of hydroxymethyl glutaryl-CoA reductase. *Proc. Natl. Acad. Sci. USA* **6**, 7797–7802 (1999).

131. B. M. Machiels, M. E. Henfling, W. L. Gerards, J. L. Broers, H. Bloemendal, F. C. Ramaekers and B. Schutte, Detailed analysis of cell cycle kinetics upon proteasome inhibition. *Cytometry* **28**, 243–252 (1997).

132. C. Wojcik, D. Schroeter, M. Stoehr, S. Wilk and N. Paweletz, An inhibitor of the chymotrypsin-like activity of the multicatalytic proteinase complex (20S proteasome) induces arrest in G_2-phase and metaphase in HeLa cells. *Eur. J. Cell Biol.* **70**, 172–178 (1996).

133. W. E. Mitch, Metabolic acidosis stimulates protein metabolism in uremia. *Miner. Electrolyte Metab.* **22**, 62–65 (1996).

134. G. Tiao, S. Hobler, J. J. Wang, T. A. Meyer, F. A. Luchette, J. E. Fischer and P. O. Hasselgren, Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle. *J. Clin. Invest.* **99**, 163–168 (1997).

135. J. L. Bailey and W. E. Mitch, Metabolic acidosis as a uremic toxin. *Semin. Nephrol.* **16**, 160–166 (1996).

136. J. L. Bailey, X. Wang, B. K. England, S. R. Price, X. Ding and W. E. Mitch, The acidosis of chronic renal failure activates muscle proteolysis in rats by augmenting transcription of genes encoding proteins of the ATP-dependent ubiquitin-proteasome pathway. *J. Clin. Invest.* **97**, 1447–1453 (1996).

137. F. Verdier, S. Chretien, O. Muller, P. Varlet, A. Yoshimura, S. Gisselbrecht, C. Lacombe and P. Mayeux, Proteasomes regulate erythropoietin receptor and signal transducer and activator of transcription 5 (STAT5) activation. Possible involvement of the ubiquitinated Cis protein. *J. Biol. Chem.* **273**, 28185–28190 (1998).

138. F. Verdier, P. Walrafen, N. Hubert, S. Chretien, S. Gisselbrecht, C. Lacombe and P. Mayeux, Proteasomes regulate the duration of erythropoietin receptor activation by controlling down-regulation of cell surface receptors. *J. Biol. Chem.* **275**, 18375–18381 (2000).

139. H. C. Drexler, Activation of the cell death program by inhibition of proteasome function. *Proc. Natl. Acad. Sci. USA* **94**, 855–860 (1997).

140. J. Delic, P. Masdehors, S. Omura, J. M. Cosset, J. Dumont, J. L. Binet and H. Magdelenat, The proteasome inhibitor lactacystin induces apoptosis and sensitizes chemo- and radioresistant human chronic lymphocytic leukaemia lymphocytes to TNF-α-initiated apoptosis. *Br. J. Cancer* **77**, 1103–1107 (1998).

141. L. W. Wu, S. Reid, A. Ritchie, H. E. Broxmeyer and D. B. Donner, The proteasome regulates caspase-dependent and caspase-independent protease cascades during apoptosis of MO7e hematopoietic progenitor cells. *Blood Cells Mol. Dis.* **25**, 20–29 (1999).

142. J. L. Herrmann, F. Briones, Jr., S. Brisbay, C. J. Logothetis and T. J. McDonnell, Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. *Oncogene* **17**, 2889–2899 (1998).

143. R. Z. Orlowski, J. R. Eswara, A. Lafond-Walker, M. R. Grever, M. Orlowski and C. V. Dang, Tumor growth inhibition induced in a murine model of human Burkitt's lymphoma by a proteasome inhibitor. *Cancer Res.* **58**, 4342–4348 (1998).

144. K. Shinohara, M. Tomioka, H. Nakano, S. Tone, H. Ito and S. Kawashima, Apoptosis induction resulting from proteasome inhibition. *Biochem. J.* **317**, 385–388 (1996).

145. P. Masdehors, S. Omura, H. Merle-Beral, F. Mentz, J. M. Cosset, J. Dumont, H. Magdelenat and J. Delic, Increased sensitivity of CLL-derived lymphocytes to apoptotic death activation by the proteasome-specific inhibitor lactacystin. *Br. J. Haematol.* **105**, 752–757 (1999).

146. H. Kitagawa, E. Tani, H. Ikemoto, I. Ozaki, A. Nakano and S. Omura, Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. *FEBS Lett.* **443**, 181–186 (1999).

147. J. Chandra, I. Niemer, J. Gilbreath, K. O. Kliche, M. Andreeff, E. J. Freireich, M. Keating and D. J. McConkey, Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. *Blood* **92**, 4220–4229 (1998).

148. L. M. Grimm, A. L. Goldberg, G. G. Poirier, L. M. Schwartz and B. A. Osborne, Proteasomes play an essential role in thymocyte apoptosis. *EMBO J.* **15**, 3835–3844 (1996).

149. R. Glas, M. Bogyo, J. S. McMaster, M. Gaczynska and H. L. Ploegh, A proteolytic system that compensates for loss of proteasome function. *Nature* **392**, 618–622 (1998).

150. E. Geier, G. Pfeifer, M. Wilm, M. Lucchiari-Hartz, W. Baumeister, K. Eichmann and G. Niedermann, A giant protease with potential to substitute for some functions of the proteasome. *Science* **283**, 978–981 (1999).

151. L. M. Grimm and B. A. Osborne, Apoptosis and the proteasome. *Results Probl. Cell. Differ.* **23**, 209–228 (1999).

152. V. A. Soldatenkov and A. Dritschilo, Apoptosis of Ewing's sarcoma cells is accompanied by accumulation of ubiquitinated proteins. *Cancer Res.* **57**, 3881–3885 (1997).

153. K. M. McGinnis, M. E. Gnegy, Y. H. Park, N. Mukerjee and K. K. Wang, Procaspase-3 and poly(ADP)ribose polymerase (PARP) are calpain substrates. *Biochem. Biophys. Res. Commun.* **263**, 94–99 (1999).

154. B. A. Teicher, G. Ara, R. Herbst, V. J. Palombella and J. Adams, The proteasome inhibitor PS-341 in cancer therapy. *Clin. Cancer Res.* **5**, 2638–2645 (1999).

155. N. Mailand, J. Falck, C. Lukas, R. G. Syljuasen, M. Welcker, J. Bartek and J. Lukas, Rapid destruction of human Cdc25A in response to DNA damage. *Science* **288**, 1425–1429 (2000).

156. F. Pajonk and W. H. McBride, Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. *Radiother. Oncol.* **59**, 203–212 (2001).

157. U. Raju, G. J. Gumin, F. Noel and P. J. Tofilon, IκBα degradation is not a requirement for the X-ray-induced activation of nuclear factor κB in normal rat astrocytes and human brain tumour cells. *Int. J. Radiat. Biol.* **74**, 617–624 (1998).

158. M. H. Seegenschmiedt and L. Keilholz, Epicondylopathia humeri (EPH) and peritendinitis humeroscapularis (PHS): Evaluation of radiation therapy long-term results and literature review. *Radiother. Oncol.* **47**, 17–28 (1998).

159. K. R. Trott and F. Kamprad, Radiobiological mechanisms of anti-inflammatory radiotherapy. *Radiother. Oncol.* **51**, 197–203 (1999).

160. B. Schopohl, D. Leirmann, L. J. Pohlit, R. Heyd, G. Strassmann, R. Bauersachs, D. Schulte-Huermann, C. G. Rahl, K. H. Manegold and H. D. Bottcher, ^{192}Ir endovascular brachytherapy for avoidance of intimal hyperplasia after percutaneous transluminal angioplasty and stent implantation in peripheral vessels: 6 years of experience. *Int. J. Radiat. Oncol. Biol. Phys.* **36**, 835–840 (1996).

161. G. Kantor, P. Van Houtte, S. Beauvois and M. Roelandts, Role of radiotherapy in benign diseases. *Cancer Radiother.* **1**, 407–416 (1997).

162. M. C. Smitt and S. S. Donaldson, Radiation therapy for benign disease of the orbit. *Semin. Radiat. Oncol.* **9**, 179–189 (1999).

163. V. Beckendorf, T. Maalouf, J. L. George, P. Bey, J. Leclere and E.

Luporsi, Place of radiotherapy in the treatment of Graves' orbitopathy. *Int. J. Radiat. Oncol. Biol. Phys.* **43**, 805–815 (1999).

164. F. Pajonk, H. Flick, H. Mittelviefhaus and J. Slanina, Postoperative pterygium prevention by radiotherapy with strontium-90 beta-rays. *Front. Radiat. Ther. Oncol.* **30**, 259–264 (1997).

165. H. Koren, G. Alth, G. M. Schenk and R. H. Jindra, Induratio penis plastica: Effectivity of low-dose radiotherapy at different clinical stages. *Urol. Res.* **24**, 245–248 (1996).

166. M. C. Joiner, P. Lambin, E. P. Malaise, T. Robson, J. E. Arrand, K. A. Skov and B. Marples, Hypersensitivity to very-low single radiation doses: its relationship to the adaptive response and induced radioresistance. *Mutat. Res.* **358**, 171–183 (1996).

167. K. Kiyomiya, S. Matsuo and M. Kurebe, Proteasome is a carrier to translocate doxorubicin from cytoplasm into nucleus. *Life Sci.* **62**, 1853–1860 (1998).

168. R. P. Koldamova, I. M. Lefterov, M. T. DiSabella and J. S. Lazo, An evolutionarily conserved cysteine protease, human bleomycin hydrolase, binds to the human homologue of ubiquitin-conjugating enzyme 9. *Mol. Pharmacol.* **54**, 954–961 (1998).

169. J. Adams, V. J. Palombella, E. A. Sausville, J. Johnson, A. Destree, D. D. Lazarus, J. Maas, C. S. Pien, S. Prakash and P. J. Elliott, Proteasome inhibitors: A novel class of potent and effective antitumor agents. *Cancer Res.* **59**, 2615–2622 (1999).

170. T. Watanabe, N. Uchiyama, I. B. Roninson, D. Cohen and P. Atadja, Altered activity of MDR reversing agents on KB3-1 cells transfected with Gly185→Val human P-glycoprotein. *Int. J. Oncol.* **17**, 579–586 (2000).

171. C. G. Lee, M. M. Gottesman, C. O. Cardarelli, M. Ramachandra, K. T. Jeang, S. V. Ambudkar, I. Pastan and S. Dey, HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. *Biochemistry* **37**, 3594–3601 (1998).

172. K. Ohkawa, T. Asakura, K. Takada, T. Sawai, Y. Hashizume, Y. Okawa and N. Yanaihara, Calpain inhibitor causes accumulation of ubiquitinated P-glycoprotein at the cell surface: Possible role of calpain in P-glycoprotein turnover. *Int. J. Oncol.* **15**, 677–686 (1999).

173. A. Frankel, S. Man, P. Elliott, J. Adams and R. S. Kerbel, Lack of multicellular drug resistance observed in human ovarian and prostate carcinoma treated with the proteasome inhibitor PS-341. *Clin. Cancer Res.* **6**, 3719–3728 (2000).

174. J. Adams, V. J. Palombella and P. J. Elliott, Proteasome inhibition: A new strategy in cancer treatment. *Invest. New Drugs* **18**, 109–121 (2000).

175. G. Hu and E. R. Fearon, Siah-1 N-terminal RING domain is required for proteolysis function, and C-terminal sequences regulate oligomerization and binding to target proteins. *Mol. Cell. Biol.* **19**, 724–732 (1999).

176. C. G. Maki, J. M. Huibregtse and P. M. Howley, *In vivo* ubiquitination and proteasome-mediated degradation of p53(1). *Cancer Res.* **56**, 2649–2654 (1996).

177. H. L. Pahl and P. A. Baeuerle, Control of gene expression by proteolysis. *Curr. Opin. Cell Biol.* **8**, 340–347 (1996).

178. M. Rolfe, M. I. Chiu and M. Pagano, The ubiquitin-mediated proteolytic pathway as a therapeutic area. *J. Mol. Med.* **75**, 5–17 (1997).

179. N. Ishida, K. Tanaka, T. Tamura, M. Nishizawa, K. Okazaki, N. Sagata and A. Ichihara, Mos is degraded by the 26S proteasome in a ubiquitin-dependent fashion. *FEBS Lett.* **324**, 345–348 (1993).

180. A. Kalmes, C. Hagemann, C. K. Weber, L. Wixler, T. Schuster and U. R. Rapp, Interaction between the protein kinase B-Raf and the alpha-subunit of the 11S proteasome regulator. *Cancer Res.* **58**, 2986–2990 (1998).

ELSEVIER

Radiotherapy and Oncology 59 (2001) 203–212

RADIOTHERAPY & ONCOLOGY

www.elsevier.com/locate/radonline

Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses

Frank Pajonk[a,*], William H. McBride[b]

[a]Department of Radiation Therapy, Radiological University Clinic, Hugstetter Strasse 55, 79106 Freiburg i. Brsg. Germany
[b]Department of Radiation Oncology, Experimental Division, UCLA School of Medicine, Los Angeles, CA, USA

Received 29 May 2000; received in revised form 7 January 2001; accepted 19 January 2001

Abstract

Background and purpose: Ionizing radiation is known to activate certain signal transduction pathways, the regulation of which could involve post-transcriptional as well as transcriptional mechanisms. One of the most important post-transcriptional pathways in eukaryotic cells is the ATP- and ubiquitin-dependent degradation of proteins by the 26s proteasome. This process controls initiation of many cellular stress responses, as well as inflammatory responses under control of the transcription factor NF-κB. The literature on the relationship between radiation and inflammation seems somewhat paradoxical. At high doses, radiation is generally pro-inflammatory. On the other hand, low dose radiation has a long history of use in the treatment of inflammatory disease. This suggests the involvement of multiple mechanisms that may operate differentially at different dose levels.

Materials and methods: In this paper, the ability of different doses of ionizing radiation to directly affect 26s proteasome activity was tested in ECV 304 cells. Proteasome activity, IκBα protein levels, and NF-κB activation were monitored.

Results: Inhibition of chymotrypsin-like 20s and 26s proteasome activity was observed immediately after low- and high-dose irradiation either of cells or purified proteasomes. The inhibitory effect was independent of the availability of the known endogenous proteasome inhibitor heat shock protein 90 (hsp90). Levels of IκBα, a physiological 26s proteasome substrate, were increased only at low doses (0.25 Gy) and unaltered at higher doses whereas only the highest doses (8 and 20 Gy) activated NF-κB.

Conclusions: We conclude that the proteasome is a direct target of ionizing radiation and suggest that inhibition of proteasome function provides a molecular framework within which low dose anti-inflammatory effects of radiation, and radiation-induced molecular responses in general, should be considered. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Gene regulation; Transcription factors; Signal transduction; Acute phase reactants; Inflammatory mediators

1. Introduction

In recent years considerable interest has been shown in the mechanisms by which ionizing radiation induces early molecular responses and how these might influence subsequent radiation-related events [8,16]. Cellular gene expression can be re-orchestrated by radiation within minutes to hours. The most frequently reported immediate early responses include up-regulation of expression of the transcription factors JUN, FOS, and NF-κB, [2,4,14,15,20,28, 43,44,47,50] and of molecules implicated in recognition and repair of damaged DNA, in responses to oxidative stress, and in cell cycle arrest and death [16]. These immediate early responses co-ordinately link radiation damage to pathways that promote wound healing and tissue remodeling [8].

Gene expression can be modified by post-transcriptional as well as transcriptional mechanisms. The former are generally the more rapid. NF-κB, which is the prime player linking radiation and other signals to inflammatory responses, is a good example. NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105). Activation of this pathway is normally achieved by phosphorylation of one of the most important inhibitors, IκBα, at two serine-sites (Ser-32 and Ser-36) by IκB kinases. This marks IκBα for polyubiquitination and subsequent degradation by the 26S proteasome. Degradation of IκBα frees NF-κB for translocation to the nucleus and activation of its target genetic programs (reviewed in [3]).

NF-κB activation in response to pro-inflammatory agents such as TNFα is a redox-sensitive process [42]. It is therefore not surprising that ionizing radiation can activate NF-κB [31] through the classical ubiquitin/proteasome-depen-

* Corresponding author.

0167-8140/01/$ - see front matter © 2001 Elsevier Science Ireland Ltd. All rights reserved.
PII: S0167-8140(01)00311-5

dent pathway [7,26], although in astrocytes and human brain tumor cells, phosphorylation of IκBα at tyrosine residues without its subsequent degeneration has been suggested as an alternate pathway [39]. In lymphocytes, NF-κB activation that is non-linear with dose has been reported [31,26], and this is the case for certain other radiation-induced molecular responses [43]. It seems likely that this non-linearity is the result of multiple mechanisms that operate differentially with dose.

Because ubiquitination and degradation of negative regulatory molecules through the proteasome is often a first critical step for activation of many molecular pathways, we have investigated the possibility the proteasome itself is a redox-sensitive target for irradiation. The 26s proteasome is responsible for the controlled ATP-and ubiquitin-dependent degradation of all short-lived [9] and 70–90% of all long-lived proteins [9,25], including key molecules in signal transduction, cell cycle control, and immune response [41]. Recently, it has become clear that this activity can be intrinsically regulated. Here, we provide evidence that the 26s proteasome complex is a direct target of ionizing radiation and that this mechanism operates functionally to inhibit activation of NF-κB at low radiation doses. It is of interest that although high dose radiation is generally pro-inflammatory [30], the history of radiation therapy for benign diseases in particular is replete with examples where ionizing radiation was used to terminate preexisting inflammatory conditions [45]. The molecular basis for this paradox is unexplained, although low radiation doses have been shown to inhibit nitric oxide production by macrophages, while high doses result in super-stimulation [18]. The relative contributions of post-transcriptional and transcriptional control mechanisms could help to explain some of the immediate early molecular effects of ionizing radiation at different dose levels.

2. Materials and Methods

2.1. Cell culture

Cultures of ECV 304 human bladder carcinoma cells (ATCC) and RAW 264.7 murine macrophages (a generous gift of Dr G. Hildebrandt, Department of Radiation Oncology, University Leipzig) were grown in 75 cm^2 flasks (Falcon) at 37°C in a humidified atmosphere at 5% CO_2. The medium used was DMEM medium (Gibco BRL) supplemented with 10 % FCS, 1 % penicillin/streptomycin (Gibco BRL), and 0.5 mg/ml fungizone (amphotericin B, Gibco BRL).

2.2. Irradiation

EVC 304 cells were trypsinized, counted and 1×10^5 cells were plated into culture dishes (Falcon, 5 cm). After 24 h plates were irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shepherd, Mark I) at a dose rate of 5.527 Gy/min. Corresponding controls were sham irradiated. Partially purified proteasome fractions were resuspended and immediately irradiated on ice. Control samples were sham irradiated.

2.3. Proteasome function assays

Proteasome function was measured as described previously [12] with some minor modifications. To obtain crude cellular extracts, cells were washed with PBS, then with buffer I (50 mM Tris (pH 7.4) 2 mM dithiothreitol (DTT), 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation ($1000 \times g$, 5 min, 4°C). Glass beads and homogenization buffer (50 mM Tris (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 min. Beads and cell debris were removed by centrifugation at $1000 \times g$ for 5 min and $10\,000 \times g$ for 20 min at 4°C.

Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. Partially purified proteasomes were prepared as described previously [22] with some minor modifications. Crude cellular extracts were subjected to ultra-centrifugation at $100\,000 \times g$ at 4°C for 3 h. The supernatant was transferred into fresh tubes and the resulting pellet was resuspended in homogenization buffer.

To measure 26s proteasome activity, 10 μg protein of crude cellular extracts or 1 μg protein of partially purified proteasomes of each sample was diluted with buffer I to a final volume of 200 μl. For assessment of 20s proteasome activity, 1 μg of protein was diluted to a final volume of 200 μl in a buffer consisting of 50 mM Tris–HCl (pH7.9), 0.5 mM EDTA and 0.05% SDS. PS-34 1 [1] was kindly provided by Julian Adams, ProScript Inc., MA and was solubilized in acidified ethanol and stored in small aliquots at a concentration of 0.5 mg/20 μl at −80°C. PS-341 was added to the reaction buffers at a final concentration of 5 μM to prove the specificity of the cleavage reaction. The fluorogenic proteasome substrate SucLLVY-MCA (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 100 μM in 1% dimethyl sulfoxide (DMSO). Proteolytic activity was continuously monitored by measuring the release of the fluorescent group 7-amido-4-methylcoumarin (AMC) in a fluorescence plate reader (Spectrafluor, Tecan, 37°C) at 380/460 nm.

2.4. Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris–HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodium vanadate). Protein concentrations were determined using the BCA protocol (Pierce) with BSA (Sigma) as standard. 50 μg of protein were electrophoresed in a SDS gel (0.1% SDS/12% polyacrylamide) and blotted to PVDF membranes at 4°C. Uniformity of loading was confirmed by Coomassie staining. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, 5%

skim milk) for 1 h at room temperature the membranes were incubated with a polyclonal antibody against human IκBα (0.5 μg/ml, Santa Cruz Biotechnologies) for 1 h at room temperature. A secondary HRP-conjugated antibody and the ECLplus system (Amersham) were used for visualization.

2.5. Cell extracts and electrophoretic mobility shift assays

For preparation of total cytosolic extracts, normal and treated cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX-buffer (20 mM HEPES (pH 7.9), 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, PMSF and aprotinin) for 30 min on ice. Lysate was centrifuged at 12 000 × *g* for 5 min at 4°C. Protein concentration was determined using the BCA protocol (Pierce) and bovine serum albumin (BSA, Sigma) as standard. 15 μg protein of the resulting supernate was incubated for 25 min at room temperature with 2 μl BSA (10 μg/μl), 2 μl dIdC (1 μg/μl), 4μl Ficoll-buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF), 2 μl buffer D + (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP40, 2 mM DTT, 0.1 mM PMSF) and 1 μl of the [γ^{32}P]-ATP labeled oligonucleotide (Promega, NF-κB: AGT-TGA GGG GAC ITT CCC AGG). For a negative control, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5 % TBE gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphor imager (Storm 860, Molecular Dynamics).

2.6. Transduction experiments

The recombinant replication-deficient adenoviruses Ad5-IκB and Ad5-LacZ were generously provided by Dr. R. Batra (UCLA/VAGLAHCS, Los Angeles, CA). The vectors had been generated and quality tested at the Vector Core at the Gene Therapy Center of the University of North Carolina, School of Medicine and are described elsewhere [27]. Ad5-IκB contains a gene for a NF-κB super-repressor IκBα under control of a CMV-promoter/enhancer. The encoded protein contains serine-to-alanine mutations in residues 32 and 36, preventing its phosphorylation, ubiquitination, and subsequent degradation by the 26s proteasome. The Ad5-LacZ is a control virus that contains the gene for β-galactosidase instead of IκBα. Transduction was performed as described previously [37]. Briefly, cells were plated into culture dishes (10 cm, Falcon). After 24 h, the medium was changed and viral vectors containing the non-phosphorable IκBα or β-galactosidase gene was added at a multiplicity of infection (MOI) of 1000. After 2 h incubation, the virus-containing medium was replaced by fresh medium and cells were incubated for additional 48 h to allow gene expression.

2.7. Drug treatment

The 26s proteasome inhibitor MG-132 (Calbiochem) was dissolved in DMSO (10 mM) and small aliquots (30 μl) were stored at −20°C. Three hours before irradiation, growth medium was replaced by medium containing MG-132 (50μM, 0.5% DMSO). Control cells were subjected to DMSO treatment alone (final concentration 0.5%). In order to activate NF-κB RAW 264.7 cells were stimulated by addition of 0.1 μg/ml lipopolysaccharide (LPS from *Eschericia coli*; Serotype 0111:B4) and 100 U/ml murine recombinant interferon-gamma to the complete medium for 6 h before irradiation.

3. Results

3.1. Ionizing radiation inhibits 20s and 26s proteasome function

There are numerous reports on the use of the ECV 304 cell line as a model for human endothelium. Although this cell line has been recently identified to be a variant of the T24 bladder carcinoma cell line [11], it mimics an endothelial phenotype. In this study, it served as model for cells in an inflammatory environment by exhibiting constitutive and inducible NF-κB and ICAM-1 activity. RAW 264.7 murine macrophages are also a well accepted model for studying inflammatory responses [18].

In order to explore the 26s proteasome as a possible direct target of ionizing radiation, cells were irradiated with different doses and incubated for 30 min, 3 or 24 h. Chymotrypsin-like 26s proteasome function was assessed in crude extracts of ECV 304 cells by the rate of release of the fluorogenic compound 7-amido-4-methylcoumarin (AMC) from the proteasome substrate Suc-LLVY-AMC with continuous monitoring.

Doses from 0.17 to 2 Gy gave a rapid, dose-dependent decrease in chymotrypsin-like 26s proteasome activity from 73% to 53% of baseline levels at 3 h after irradiation. Higher radiation doses of 4, 8, or 20 Gy did not increase the extent of inhibition (Fig. 1A). Inhibition was observed as early as 30 min after irradiation and had not completely recovered by 24 h (data not shown).

The possible contribution of heat-shock protein 90 (hsp90) to the inhibitory effect, which has been described as a preformed endogenous inhibitor of proteasome function [46], was also investigated. Binding of hsp90 to any of its dimerization partners can be selectively disrupted by the benzoquinone ansamycin geldanamycin [24]. Preincubation of ECV 304 cells with geldanamycin increased proteasome activity, in keeping with it blocking hsp90 activity, but it failed to prevent radiation-induced proteasome inhibition when compared to non-irradiated geldanamycin-treated controls, excluding the possibility of radiation-

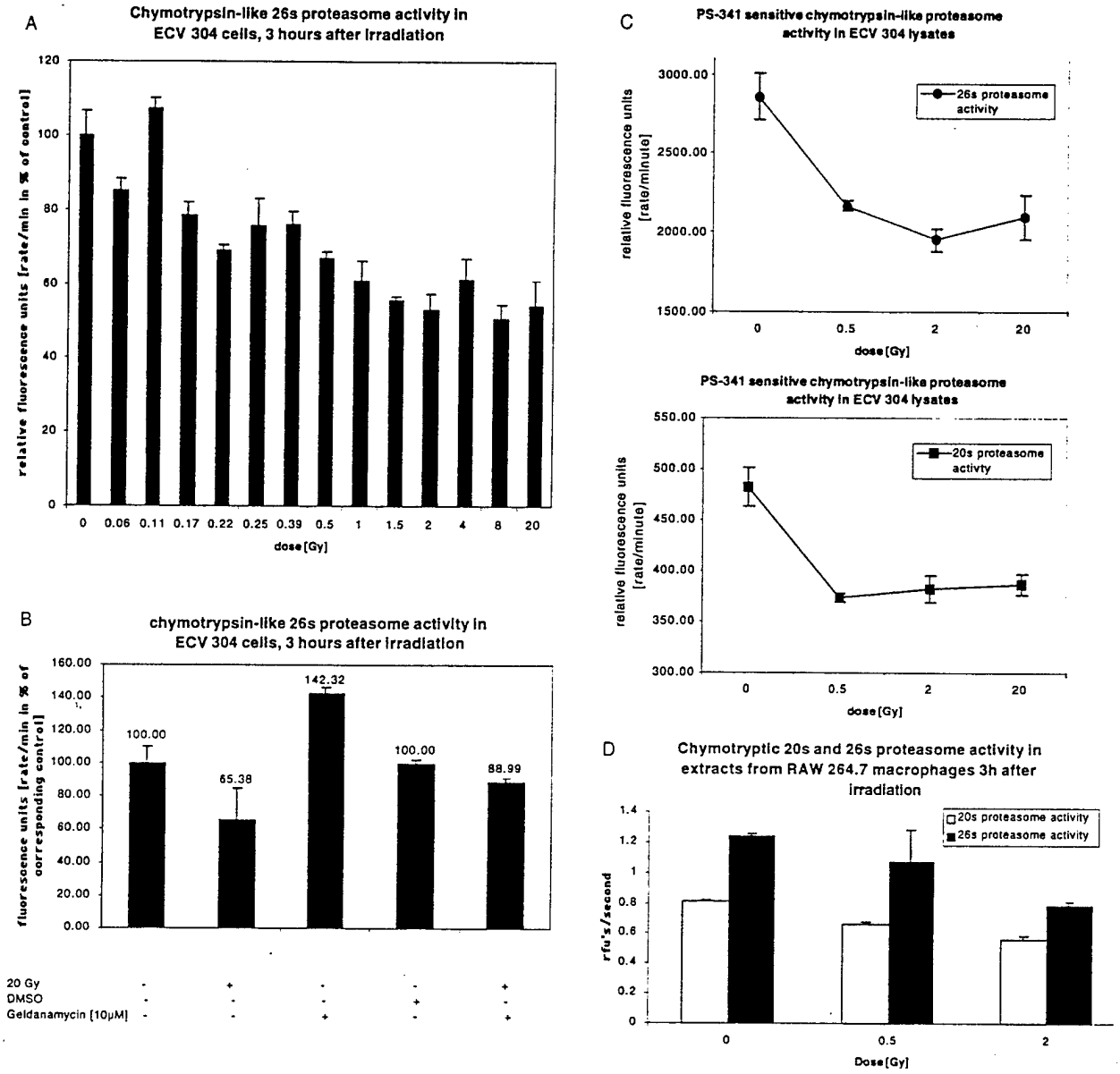


Fig. 1. (A) 26s proteasome function assay for chymotrypsin-like proteolytic activity in crude lysates of ECV 304 cells. 3 h after application of different dose of ionizing irradiation ($n = 4$, means ± standard deviation). Low dose ionizing radiation (0.17–2 Gy) caused a significant reduction of 26s proteasome cleavage activity (2 Gy: 52.97% ± 4.3 of untreated control cells, $P < 0.001$, Student's t-test). Application of higher doses (4–20 Gy) could not further enhance this effect. (B) Chymotrypsin-like 26s proteasome activity in crude lysates of ECV 304 cells, 3 h after irradiation ($n = 4$, means ± standard deviation). Application of 2Gy ionizing radiation significantly reduced the proteolytic activity of the complex (65.4% ± 6.7, $P < 0.001$, Student's t-test) when compared to sham irradiated controls. Inactivation of hsp90 by pretreatment of cells (30 min before irradiation) with geldanamycin (10 µM) increased the baseline cleavage rate 1.4 fold (142.3% ± 4.4, $P < 0.001$, Student's t-test, compared to DMSO treated controls), but failed to prevent radiation induced proteasome inhibition. (C) 26s and 20s proteasome activity in purified proteasome preparations from ECV 304 cells. The purified complex was irradiated on ice. The baseline activity of the 26s proteasome complex was about 6-fold higher than the activity of the 20s proteasome complex. Application of 0.5, 2 and 20 Gy immediately and significantly inhibited 26s and 20s proteasome activity when compared to sham irradiated control preparations (upper graph, 26s proteasome activity: 0.5 Gy: 75.8% ± 1.1, $P < 0.01$; 2 Gy: 68.3% ± 2.5, $P < 0.01$; 20 Gy: 73.4% ± 5.1, $P < 0.05$, Student's t-test. Lower graph, 20s proteasome activity: 0.5 Gy: 77.4% ± 0.9, $P < 0.01$; 2 Gy: 79.3% ± 2.7, $P < 0.01$; 20 Gy: 80.2% ±2.2, $P < 0.01$, Student's t-test ($n = 2$, means ± standard deviation).

induced liberation of this inhibitor from intracellular binding partners as a mechanism of inhibition (Fig. 1B).

The contribution of cytoplasmic components to the inhibitory effect was further excluded by showing that irradiation of purified 26s proteasome complexes from ECV 304 cells on ice directly inhibited their activity. Application of 0.5 to 20 Gy caused a highly significant reduction of both 26s and 20s chymotrypsin-like proteasome activity (Fig. 1C; 2 Gy, 26s: 68.3 ± 2.5%; 20s: 79.3 ± 2.7%). The pelleted complexes that were used in these experiments contained practically all of the cellular chymotrypsin-like cleavage activity, and activity could be inhibited by the

specific proteasome inhibitor PS-341 [1] at a concentration of 5 μM. It seems therefore that the effect of radiation on the proteasome is direct.

In order to show that this effect is not restricted to human ECV cells, we extended our experiments to murine RAW 264.7 macrophages. RAW 264.7 were irradiated with 0.5 and 2 Gy and incubated for 3 h at 37°C. Control cells were sham-irradiated. Chymotryptic 20s and 26s proteasome activity was monitored in subsequently prepared lysates at different substrate concentrations (25, 50, 100 and 200 μM SucLLVY-AMC). 20s as well as 26s proteasome activity was reduced in a dose-dependent manner (Fig 1D/F; 20s: 0.5 Gy 81.5 ± 1.7%, 2 Gy 69.1 ± 2.6%; 26s: 0.5 Gy 86.5 ± 17.3%, 2 Gy 63.5 ± 1.9%, 200 μM SucLLVY-MCA).

3.2. Ionizing radiation stabilizes target molecules of the 26s proteasome

Activation of NF-κB in response to a variety of stimuli is generally characterized by disappearance of IκB as a result of its phosphorylation and subsequent proteasome-mediated degradation. Proteasome inhibitors can attenuate NF-κB induction by blocking this degradative pathway [26]. The observed radiation-induced inhibition of proteasome function by radiation is at odds with radiation-induced NF-κB activation. We therefore considered the possibility that radiation has both inhibitory and enhancing effects on NF-κB activation, that multiple mechanisms operate, and that the outcome depends on radiation dose.

To investigate the relationship between radiation-induced inhibition of proteasome function and expression of IκBα, immunoblotting studies were performed using a polyclonal antibody against IκBα with total cellular extracts of ECV 304. IκBα expression was increased 30 min after irradiation with the lowest doses (0.25–0.5 Gy), in keeping with an effect of proteasome inhibition (Fig. 2), but was essentially not altered after higher doses.

3.3. Radiation-induced NF-κB activity

To examine NF-κB activity, a gel shift assay was used 3 h after various radiation doses. Analysis of eight independent gel-shift experiments showed no significant change in NF-κB DNA-binding activity after 0.25 Gy (1.02-fold ± 0.06) or 0.5 Gy (1.03-fold ± 0.03). Irradiation with 1, 1.5, 2 and 4 Gy caused a slight consistent, but not significant, elevation in NF-κB activity. Significant increases were achieved only after application of 8 Gy (1.24-fold ± 0.03, $P < 0.01$, Student's *t*-test) or 20 Gy (1.3-fold ± 0.05, $P < 0.001$, Student's *t*-test) (Fig. 3A,B).

These experiments with ECV 304 cells dealt with constitutive NF-κB DNA-binding activity, rather than with an induced inflammatory response. In order to investigate this aspect of the effect of ionizing radiation on inflammatory responses, we activated NF-κB in RAW 264.7 macrophages using LPS and IFN-α. Gel-shifts from total cellular lysates prepared 3 h after irradiation revealed a dose dependent decrease in NF-κB DNA-binding activity (Fig. 3C), showing that radiation could suppress, as well as activate, NF-κB activity.

The dependence of NF-κB activation by radiation on proteasome activity and IκB turnover has been questioned [39], largely because of observations that IκBα expression does not decrease in the same manner as it does in response to TNF stimulation [32]. At least in ECV 304, NF-κB activation still appears to depend on this pathway, although the kinetics of IκB turnover may be altered. Expression of the 'super repressor' IκBα mutant, which contains alanines at positions 32 and 36, prevented constitutive and radiation-induced activation of NF-κB (Fig. 4A), as did treatment with MG-132 (Fig. 4B) at doses that inhibited proteasome activity completely [36]. Transduction with the β-galactosidase gene did not decrease, and in fact increased, NF-κB activity, in keeping with the pro-inflammatory nature of the adenoviral vector, and NF-κB activity was still inducible by irradiation (Fig. 4A).

4. Discussion

Knowledge about molecular mechanisms activated after application of ionizing radiation is a key to understanding early and late side effects of radiation therapy. Also, identification of the sub-cellular targets of ionizing radiation might uncover new approaches to improve treatment outcome and to minimize toxicity.

Certain aspects of the molecular response to ionizing radiation can appear paradoxical. On one hand, signal transduction pathways leading to production of pro-inflammatory cytokines and cell adhesion molecules can be rapidly activated [19]. A major player in this response is the transcription factor NF-κB [26] (reviewed in [29]). One the other hand, ionizing radiation has anti-inflammatory and immunosuppressive effects in certain situations and the use of ionizing radiation in the treatment of benign inflammatory as well as benign hyperproliferative conditions is almost as old as the knowledge about radiation itself [48].

At present there are about 150 stimuli known to activate the NF-κB, including ionizing radiation, TNFα, and LPS, each causing transcriptional activation of about the same number of NF-κB dependent genes (reviewed in [34]). The exact mechanism of how radiation activates NF-κB is not known, but the initial steps are redox sensitive (reviewed in [42]). The classic mechanism of serine phosphorylation and degradation of IκB through the ubiquitin proteasome system [26] may be involved, although alternative pathways have been reported [39]. Some of the more paradoxical effects of radiation could be explained by multiple mechanisms operating at different dose levels to give what are essentially non-linear responses. Furthermore, many radiation-induced immediate early gene effects could be explained by alterations in proteasome processing.

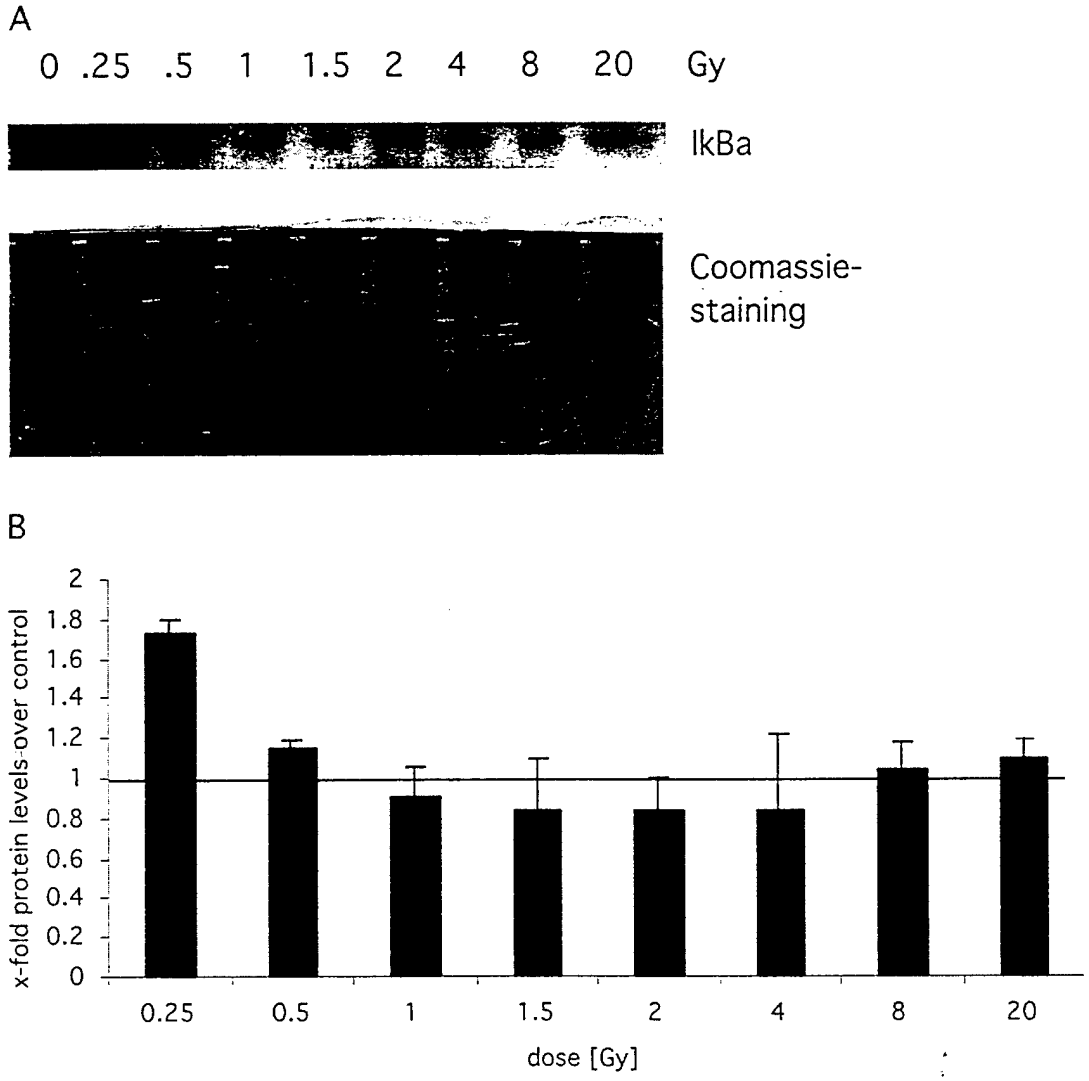
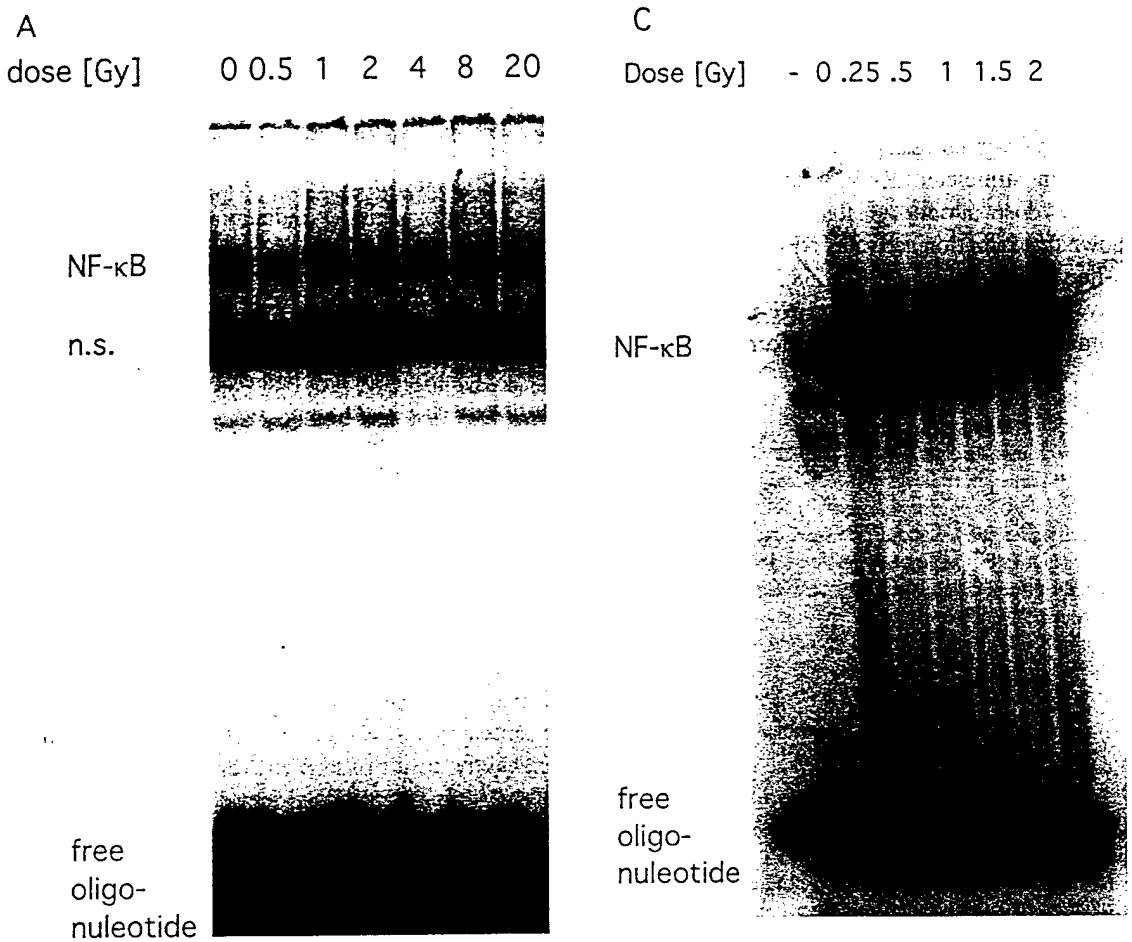


Fig. 2. (A,B) Western blot analysis of total cellular lysates from ECV 304 cells 30 min after irradiation using a polyclonal antibody against IκBα. Equity of loading was confirmed by coomassie staining. Application of 0.25 Gy significantly increased IκBα protein levels (173.2% ± 6.2, $P < 0.05$, Student's t-test) while higher doses did not cause significant changes ($n = 2$, means ± standard deviation).

We have demonstrated that the proteolytic activity of the 20s and 26s proteasome was directly compromised by ionizing radiation and that this inhibitory effect was not species- or cell-type-dependent. The observed effect was immediate and lasted for at least 24 h. After low dose (<2 Gy) irradiation of cells, 26s proteasome function declined to about 60% of baseline levels. Higher doses did not further decrease the activity. This inhibitory effect was also observed when purified proteasome preparations were irradiated on ice, indicating the involvement of a radiochemical rather than a biological mechanism. Reinheckel et al. [40] have reported that proteasome activity is sensitive to oxidative damage following direct addition of hydrogen peroxide. About 60% inhibition was achieved with concentrations of hydrogen peroxide up to 1 mM. Higher hydrogen peroxide concentrations did not lead to a further significant decrease of proteolytic activity. This suggests that the proteasome is sensitive to redox changes. Our finding that proteasome activity is inhibited by low concentrations of *N*-acetylcysteine (Pajonk, unpublished) is consistent with this view. The finding that radiation affected both 26s and 20s proteasome activity, suggests that ionizing radiation inhibits the catalytic sites in the 20s core complex, though effects on the regulatory cap molecules can not be excluded. It is also possible that within the cell additional mechanisms play a role, although our experiments tend to exclude participation of the known endogenous inhibitor hsp90.

In ECV 304 cells, expression of IκBα, the most important member of the IκB molecular family, was enhanced after application of low doses of radiation that blocked protea-



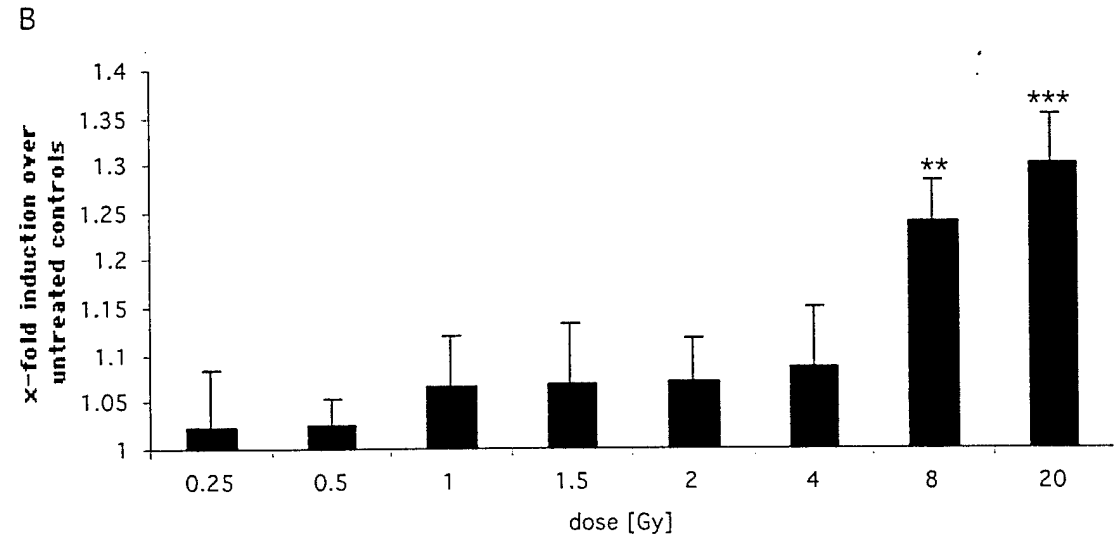


Fig. 3. (A) Representative gel-shift experiment and densometric analysis (B) of NF-κB DNA-binding activity in lysates from ECV 304 cells 3 h after irradiation (means ± standard deviation from eight independent experiments). Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2: untreated control cells. Lane 3 to 10: NF-κB activity after application of 0.25–20 Gy ionizing radiation. NF-κB DNA-binding activity in response to ionizing radiation is retarded and increases over baseline levels only after application of 8 and 20 Gy. (C) Representative gel-shift experiment of NF-κB DNA-binding activity in lysates from RAW 264.7 murine macrophages 3 h after irradiation. Cells have been pre-stimulated using LPS- and IFN-γ-supplemented media for 6 h. Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2–7: NF-κβ activity after application of 0 (lane 2), 0.25 (lane 3), 0.5 (lane 4), 1 (lane 5), 1.5 (lane 6) and 2 Gy (lane 7) of ionizing radiation.

some activity and was not decreased after higher doses, even though they caused NF-κB activation. Inhibition of proteasome activity may explain the failure of others to demonstrate decreases in IκBα levels after irradiation [39].

Radiation doses up to 4 Gy failed to significantly alter constitutive NF-κB DNA-binding activity in ECV cells and decreased activity in stimulated RAW macrophages, in keeping with a low dose inhibitory effect. A number of studies have shown that radiation activates NF-κB in a fashion that is non-linear with dose [17,26,31,38,39], as is the case in our study. Previous reports showed NF-κB activation to be maximal at 0.5 Gy in EBV-transformed 244B human lymphoblastoid cells [31,38], while primary human B cells show a strong activation of NF-κB at 15 Gy [49].

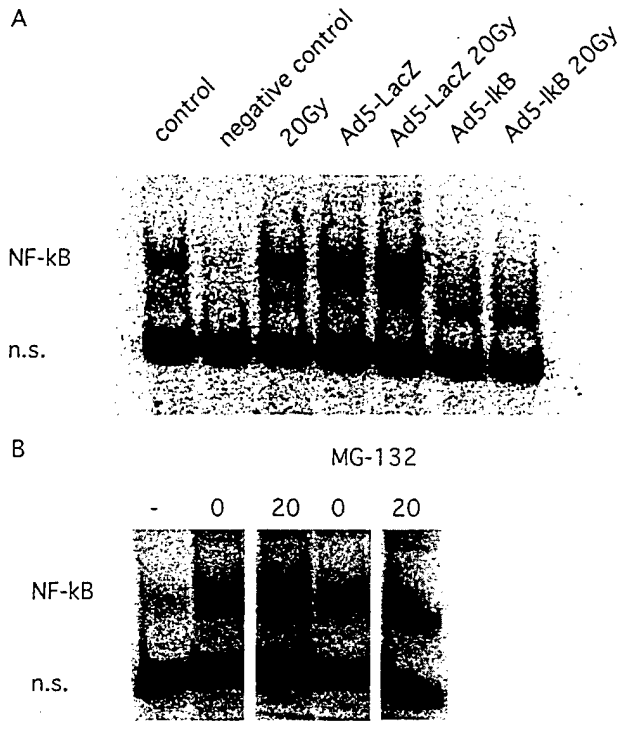


Fig. 4. (A) Representative gel-shift experiment with lysates of ECV 304 cells. Cells were transduced with an adeno-viral vector containing either a gene for B-galactosidase (Ad5-LacZ) or a gene for an IκB superrepressor (Ad5-IκB). The IκB superrepressor has serine-to-alanine mutations at position 32 and 36 and is thus unphosphorable by IκB kinases. 48 h after transduction the cell were treated with 20 Gy ionizing radiation and incubated for additional 3 h. Constitutive and radiation induced activation of NF-κB was blocked by the expression of the IκB superrepressor but not by the B-galactosidase gene. Additionally, transduction with the Ad5-LacZ caused an increase in constitutive NF-κB activity. Lane 1: untreated control cells. Lane 2: negative control: the unlabeled oligonucleotide was added in 50-fold excess to show specificity of binding. Lane 3: non-transduced cells after 20 Gy. Lane 4 and 5: cells transduced with Ad5-LacZ after 0 and 20 Gy. Lane 6 and 7: cells transduced with Ad5-IκB after 0 and 20 Gy. (B) Gel-shift experiment for NF-κB in ECV 304 cells 3 h after application of ionizing irradiation with and without preincubation with the proteasome inhibitor MG-132 (50 μM, 3 h). Proteasome inhibition prevents constitutive and radiation induced activation of NF-κB. Lane 1: negative control; the unlabeled oligonucleotide was added 50-fold excess to show specificity of binding. Lane 2 and 3: NF-κB activity in ECV 304 cells 3 h after irradiation with 0 Gy (2) or 20 Gy (3). Lane 4 and 5: NF-κB DNA-binding activity in ECV 304 cell 3 h after irradiation with 0 Gy (4) or 20 Gy (5) and preincubation with the proteasome inhibitor MG-132.

NF-κB activation following ionizing radiation and other stimuli generally requires degradation of IκBα as an obligatory step [23,26]. NF-κB activation without degradation of IκBα has been reported for UV radiation [6,26] and anoxia [21], and in a recent study following exposure of brain cells to ionizing radiation [39]. The latter study suggested tyrosine-phosphorylation of IκB as a mechanism, although this was not demonstrated directly and the effects of erbstatin could have been on proteins upstream of IκB as has been described for NF-κB activation after TNFα treatment [32]. In our study, classic processing of serine phosphorylated IκB through the proteasome following irradiation seems to be responsible for NF-κB activation. NF-κB activation was blocked by addition of the proteasome inhibitor MG-132, as Hallahan et al. [17] also reported. In addition, the IκB super-repressor gene, which contains serine-to-alanine mutations at position 32 and 36, preventing its serine-phosphorylation by IκB-kinases and subsequent degradation by the 26s proteasome, blocked constitutive and radiation-induced NF-κB. Transduction with the vector carrying the gene for β-galactosidase actually activated NF-κB in ECV 304 cells. This activation could be the result of an ER-overload response [33,35] or a direct pro-inflammatory effect of the vector as described previously [10].

Although the classic pathway of NF-κB activation appeared to operate, like Raju et al. [39], we did not find a decrease in IκBα after ionizing radiation. A possible explanation is that the increased rate of degradation after phosphorylation was offset by inhibition of the rate of proteasome degradation, resulting in little change in expression. Thus, the dose dependency of the response is most likely determined at the level of phosphorylation and the outcome is impacted at all doses by inhibition of the rate of degradation and alteration in the kinetics of IκBα turnover. Further studies are needed to determine if this is the case.

Maximal proteasome inhibition by radiation in this study was achieved even with low doses such as have been used in the treatment of benign disorders. Furthermore, our findings might provide a theoretical basis for empirically established daily fractions of 1.8–2 Gy in the treatment of malignant disorders, resulting in maximal therapeutic benefit with minimized acute reactions. It is intriguing to note that, in addition to NF-κB, expression of p53, p21, p27, pRb, and several cyclins is regulated by proteasomal degradation [41]. The inhibitory effect of ionizing radiation on proteasome activity therefore should be taken into account when considering many of the radiation induced molecular changes, including those leading to cell cycle arrest. Also, since the proteasome is responsible for much of the processing of antigen for presentation by MIHC-I molecules (reviewed in [5,13]), part of the immunosuppressive effects of radiation could be mediated by through this pathway.

References

[1] Adams J, et al. Potent and selective inhibitors of the proteasome: dipeptidyl boronic acids. Bioorg Med Chem Lett 1998;8(4):333–338.

[2] Anderson A, Woloschak GE. Cellular proto-oncogene expression following exposure of mice to gamma rays. Radiat Res 1992;130(3):340–344.

[3] Baeuerle PA, Baltimore D. NF-kappa B: ten years after. Cell 1996;87(1):13–20.

[4] Basu S, et al. The DNA-dependent protein kinase participates in the activation of NF kappa B following DNA damage. Biochem Biophys Res Commun 1998;247(1):79–83.

[5] Belich MP, Trowsdale J. Proteasome and class I antigen processing and presentation. Mol Biol Rep 1995;21(1):53–56.

[6] Bender K, et al. Sequential DNA damage-independent and -dependent activation of NF-kappaB by UV. EMBO J 1998;17(17):5170–5181.

[7] Brach MA, et al. Ionizing radiation induces expression and binding activity of the nuclear factor kappa B. J Clin Invest 1991;88(2):691–695.

[8] Chiang C-S, Hong J-H, Stalder A, Sun J-RH, Withers HR, McBride WH. Delayed molecular responses to brain irradiation. Int J Radiat Biol 1997;72(1):45–53.

[9] Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell 1994;79(1):13–21.

[10] Clesham GJ, et al. High adenoviral loads stimulate NF kappaB-dependent gene expression in human vascular smooth muscle cells. Gene Therapy 1998;5(2):174–180.

[11] Dirkis WG, MacLeod RA, Drexier HG. ECV 304 (endothelial) is really T24 (bladder carcinoma): cell line cross-contamination at source [letter]. In Vitro Cell Dev Biol Anim 1999;35(10):558–559.

[12] Glas R, et al. A proteolytic system that compensates for loss of proteasome function. Nature 1998;392(6676):618–622.

[13] Groettrup M, et al. Peptide antigen production by the proteasome: complexity provides efficiency. Immunol Today 1996;17(9):429–435.

[14] Gubits RM, Geard CR, Schiff PB. Expression of immediate early genes after treatment of human astrocytoma cells with radiation and taxol. Int J Radiat Oncol Biol Phys 1993;27(3):637–642.

[15] Hallahan DE, et al. Radiation signaling mediated by Jun activation following dissociation from a cell type-specific repressor. J Biol Chem 1993;268(7):4903–4907.

[16] Hallahan DE, et al. Spatial and temporal control of gene therapy using ionizing radiation. Nat Med 1995;1(8):786–791.

[17] Hallahan DE, Virudachalam S, Kuchibhotla J. Nuclear factor kappaB dominant negative genetic constructs inhibit X-ray induction of cell adhesion molecules in the vascular endothelium. Cancer Res 1998;58(23):5484–5488.

[18] Hildebrandt G, et al. Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. Int J Radiat Biol 1998;74(3):367–378.

[19] Hong JH, et al. Induction of acute phase gene expression by brain irradiation. Int J Radiat Oncol Biol Phys 1995;33(3):619–626.

[20] Hong JH, et al. Induction of c-fos and junB mRNA following in vivo brain irradiation. Brain Res Mol Brain Res 1997;48(2):223–228.

[21] Imbert V, et al. Tyrosine phosphorylation of I kappa B-alpha activates NFkappa B without proteolytic degradation of I kappa B-alpha. Cell 1996;86(5):787–798.

[22] Kanayama HO. Demonstration that a human 26s proteolytic complex consists of a proteasome and multiple associated protein components and hydrolyzes ATP and ubiquitin-ligated proteins by closely linked mechanisms. Eur J Biochem 1992;206(2):567–578.

[23] Karin M. How NF-kappaB is activated: the role of the IkappaB kinase (IKK) complex. Oncogene 1999;18(49):6867–6874.

[24] Lawson B, Brewer JW, Hendershot LM. Geldanamycin, an hsp90/GRP94-binding drug, induces increased transcription of endoplasmic reticulum (ER) chaperones via the ER stress pathway. J Cell Physiol 1998;174(2):170–178.

[25] Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem 1996;271(44):27280–27284.

[26] Li N, Karin M. Ionizing radiation and short wavelength UV activate NFkappaB through two distinct mechanisms. Proc Nat Acad Sci USA 1998;95(22):13012–13017.

[27] Limuro Y, et al. NFkappaB prevents apoptosis and liver dysfunction during liver regeneration. J Clin Invest 1998;101(4):802–811.

[28] Martin M, et al. Preferential induction of c-fos versus c-jun protooncogene during the immediate early response of pig skin to gamma-rays. Cancer Res 1993;53(14):3246–3249.

[29] May MJ, Ghosh G. Signal transduction through NF-kappa B. Immunol Today 1998;19(2):80–88.

[30] McBride WH, Chiang CS, Hong JH, Withers HR. Molecular and cellular responses of the brain to radiotherapy. In: Hortobagyi DKaG, editor. Current clinical topics in cancer chemotherapy, Cambridge, MA: Blackwell Science Inc, 1997.

[31] Mohan N, Meltz ML. Induction of nuclear factor kappa B after low-dose ionizing radiation involves a reactive oxygen intermediate signaling pathway. Radiat Res 1994;140(1):97–104.

[32] Natarajan K, et al. Protein tyrosine kinase inhibitors block tumor necrosis factor-induced activation of nuclear factor-kappaB, degradation of IkappaBalpha, nuclear translocation of p65, and subsequent gene expression. Arch Biochem Biophys 1998;352(1):59–70.

[33] Pahl HL, Baeuerle PA. The ER-overload response: activation of NFkappa B. Trends Biochem Sci 1997;22(2):63–67.

[34] Pahl HL. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene 1999;18(49):6853–6866.

[35] Pahl HL, et al. Activation of transcription factor NF-kappaB by the adenovirus E3/19K protein requires its ER retention. J Cell Biol 1996;132(4):511–522.

[36] Pajonk F, Pajonk K, McBride W. Apoptosis and radisensitization of Hodgkin's cells by proteasome inhibition. Int J Radiat Oncol Biol 2000;47(4):1025–1032.

[37] Pajonk F, Pajonk K, McBride WH. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. J Natl Cancer Inst 1999;91(22):1956–1960.

[38] Prasad AV, et al. Activation of nuclear factor kappa B in human lymphoblastoid cells by low-dose ionizing radiation. Radiat Res 1994;138(3):367–372.

[39] Raju U, et al. IkappaBalpha degradation is not a requirement for the X-ray induced activation of nuclear factor kappaB in normal rat astrocytes and human brain tumour cells. Int J Radiat Biol 1998;74(5):617–624.

[40] Reinheckel T, et al. Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochem J 1998;335(Pt 3):637–642.

[41] Rolfe M, Chiu MI, Pagano M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. J Mol Med 1997;75(1):5–17.

[42] Schreck R, Albermann K, Baeuerle PA. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun 1992;17(4):221–237.

[43] Sherman ML, et al. Ionizing radiation regulates expression of the c-jun protooncogene. Proc Natl Acad Sci USA 1990;87(15):5663–5666.

[44] Syljuasen RG, Hong JH, McBride WH. Apoptosis and delayed expression of c-jun and c-fos after gamma irradiation of Jurkat T cells. Radiat Res 1996;146(3):276–282.

[45] Trott KR. Therapeutic effects of low radiation doses. Strahienther Onkol 1994;170(1):1–12.

[46] Tsubuki S, Saito Y, Kawashima S. Purification and characterization of an endogenous inhibitor specific to the Z-Leu-Leu-Leu-MCA degrading activity in proteasome and its identification as heat-shock protein 90. FEBS Lett 1994;344(2-3):229–233.

[47] Weichselbaum RR, et al. Radiation induction of immediate early genes: effectors of the radiation-stress response. Int J Radiat Oncol Biol Phys 1994;30(1):229–234.

[48] Williams FH. The Roentgen rays in medicine and surgery as an aid in diagnosis and as a therapeutic agent; designed for the use of practitioners and students. 3rd ed. with enl. appendix. New York, London: Macmillan & Co. Ltd, 1903 xxxiii, 11. 757.

[49] Wilson RE, et al. Early response gene signalling cascades activated by ionising radiation in primary human B cells. Oncogene 1993;8(12):3229–3237.

[50] Woloschak GE, Felcher P, Chang-Liu CM. Combined effects of ionizing radiation and cycloheximide on gene expression. Mol Carcinog 1995;13(1):44–49.

MILITARY MEDICINE, 167, Suppl. 1:066, 2002

NF-κB, Cytokines, Proteasomes, and Low-Dose Radiation Exposure

Guarantor: William H. McBride, DSc
Contributors: Frank Pajonk, MD*; Chi-Shuin Chiang, PhD†; Ji-Rong Sun, PhD*; William H. McBride, DSc*

Ionizing radiation shares with proinflammatory cytokines a pathway that involves reactive oxygen species and activation of the redox-sensitive nuclear transcription factor NF-κB, which leads to expression of inflammatory and cell survival programs. NF-κB activation normally requires phosphorylation of its inhibitor IκB and the inhibitor's subsequent degradation by the proteasome. Nonlinear dose-response curves have been reported for both radiation-induced cytokines and NF-κB and IκB expression with maximum exposures of less than 2 Gy and greater than 4 Gy, respectively. Radiation-inhibited proteasomes function over a wide dose range, suggesting that the proteasome is a redox-sensitive target for radiation that may function along with transcription to cause nonlinear dose-response relationships for early expression of many molecules, including NF-κB and cytokines. These pathways are relevant to low-dose radiation effects, adaptive responses, and carcinogenesis.

Introduction

Numerous reports indicate that radiation alters gene expression.[1] Few studies have examined responses systematically over a wide dose range, but there is evidence that certain responses can be elicited by doses of less than 1 Gy, whereas others require higher doses.[2,3] The spectrum of radiation-induced genes can be viewed within the functional context in which they might operate.[4] Thus, molecules that act as immediate early gene products or that signal cell cycle arrest or apoptosis are commonly induced. In addition, cytokines and cell adhesion molecules are expressed that serve to generate inflammation, tissue repair, and recovery—in other words, a wound-healing response. Associated functions of these molecules may include priming surrounding cells for adaptive survival responses, inducing genomic instability, and mediating radiation injury.

Ionizing radiation displays an interesting homology with certain cytokines and stress signals that has its roots in their common use of reactive oxygen species (ROS), which most likely accounts for the cross-talk between these signals. Two possible but diametrically opposed outcomes of ROS-mediated pathways are (1) induction of apoptosis and (2) development of resistance to further ROS effects. The outcome depends on the molecular wiring intrinsic to the cell, the magnitude of the signal, and other variables.

Integral to cellular ROS responses is NF-κB. NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered in the cytosol by inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105).[5] The classic pathway for activation of NF-κB is by phosphorylation of the inhibitor IκBα by IκB kinases, which marks IκBα for polyubiquitination and subsequent degradation by the 26S proteasome. The pathway has been reported to protect cells from apoptotic death,[6,7] but in some situations it is associated with apoptosis.[8] NF-κB activation has been reported after irradiation of human lymphoblastoid cells at doses as low as 0.25 to 2.0 Gy; the maximum is after 50 cGy[9] and ROS have been implicated.[10]

Further research is needed to determine what the target is for these low-dose radiation effects, how it differs from the target at higher doses, and how it relates to induction of pro-inflammatory cytokines and stress molecules. In this paper we propose the proteasome as a target for radiation that is particularly important in the low-dose range. The proteasome is responsible for degradation of short-lived regulatory molecules in the cell, including IκB, p21, AP-1, p53, and cyclins.[11] Many radiation-induced molecules are targeted for degradation through this mechanism, and inhibition of proteasome function could lead to their up-regulation. Working in concert with transcription, this mechanism could result in nonlinear dose-response curves for radiation-induced molecular expression.

Experimental Design and Results

In C3H/HeN mice, lung irradiation induced tumor necrosis factor-α, interleukin (IL) 1α, IL-1β, and IL-6 mRNA expression, as assessed by an RNase protection assay (Fig. 1). IL-2, IL-3, IL-4, IL-5, and interferon-γ levels were barely detectable. The responses were dose and time dependent. The dose response was nonlinear with both lower and higher doses being more effective than clinically relevant 2 Gy doses. Responses subsided within 24 hours.

Nonlinear dose- and time-dependent responses were also seen for NF-κB after irradiation of ECV304 cells (F. Pajonk and W.H. McBride, unpublished data). At 3 hours after irradiation, gel shift analyses showed activation of NF-κB after doses of 1 to 20 Gy with a sharp increase above 4 Gy. IκBα levels, measured by Western blot 30 minutes after irradiation, were elevated in the low-dose range of 25 to 50 cGy, around 2 Gy, and after high doses. Because early NF-κB activation does not require protein synthesis,[5] nontranscriptional mechanisms must be affected by irradiation. To test proteasome involvement, we measured activity in extracts of irradiated cells using a fluorogenic substrate specific for chymotrypsin-like activity.[12] The results showed that cellular irradiation decreased 26S proteasome activity even when the radiation doses were in the low range of 25 to 60 cGy (Fig. 2).

Discussion

Radiation therapy was once used more for the treatment of nonmalignant disease than for cancer treatment.[13] Relatively

*Roy E. Coats Laboratories, Department of Radiation Oncology, University of California Los Angeles, Los Angeles, CA 90064.
†Department of Atomic Science, Tsing Hua University, Hsinchu, Taiwan.
This manuscript was received for review February 2001. The revised manuscript was accepted for publication in November 2001.
Reprint & Copyright © by Association of Military Surgeons of U.S., 2002.

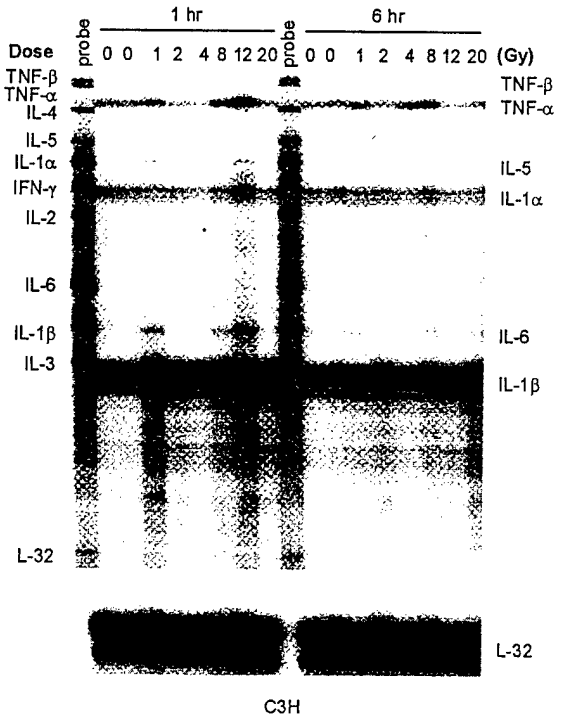


Fig. 1. Effect of lung irradiation on cytokine expression measured by RNase protection assay.

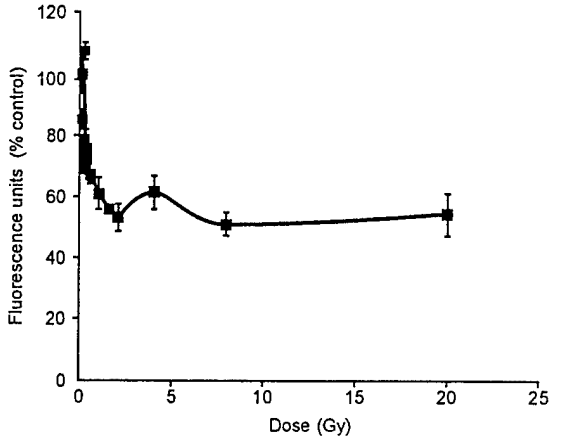


Fig. 2. Inhibition of proteasome activity 3 hours after irradiation as measured by cleavage of a fluorogenic substrate specific for chymotrypsin-like activity.

low radiation doses were frequently used to treat a variety of inflammatory and hyperproliferative conditions. This putative anti-inflammatory response contrasts with the proinflammatory response observed after higher radiation doses. The data presented here and by others[9,10] demonstrate a nonlinear dose-response curve for induction of NF-κB and proinflammatory cytokines, with a minimum of approximately 2 Gy. This dose may have been chosen for clinical use in part because of its relatively low proinflammatory potential. Both lower and higher doses were better able to stimulate these pathways.

The mechanisms underlying nonlinear radiation dose responses are complex. Gene expression is controlled at several different levels. In addition to transcriptional activation, mRNA and protein stability are under regulatory control. Many, if not all, of the short-lived regulatory proteins induced by radiation are degraded through the proteasome pathway.[11]

Conclusion

In this study, we report that radiation, even at doses as low as 50 cGy, slows the rate of degradation of fluorogenic peptides by proteasome-rich extracts. This suggests that the proteasome itself is a redox-sensitive target for radiation. The relative contributions of post-transcriptional and transcriptional control mechanisms may vary at different dose levels, which may explain the nonlinear dose- and time-response curve that is seen for some radiation-induced pathways. The findings also suggest that the proteasome might be a novel target for modification of radiation responses.

References

1. Weichselbaum RR, Hallahan DE, Sukhatme V, Dritschilo A, Sherman ML, Kufe DW: Biological consequences of gene regulation after ionizing radiation exposure. J Natl Cancer Inst 1991; 83: 480–4.
2. Fornace AJ Jr, Amundson SA, Bittner M, et al: The complexity of radiation stress responses: analysis by informatics and functional genomics approaches. Gene Exp 1999; 7: 387–400.
3. Amundson SA, Do KT, Fornace AJ Jr: Induction of stress genes by low doses of gamma rays. Radiat Res 1999; 152: 225–31.
4. McBride WH, Chiang CS, Hong JH, Withers HR: Molecular and cellular responses of the brain to radiotherapy. In Current Clinical Topics in Cancer Chemotherapy. Edited by Khayat D, Hortobagyi G. Cambridge, MA, Blackwell Science, Inc., 1997.
5. Baeuerle PA, Baltimore D: NF-κB: ten years after. Cell 1996; 87: 13–20.
6. Beg AA, Baltimore D: An essential role for NF-κB in preventing TNF-α-induced cell death. Science 1996; 274: 782–4.
7. Baichwal VR, Baeuerle PA: Activate NF-κB or die? Curr Biol 1997; 7: R94–6.
8. Kasibhatla S, Brunner T, Genestier L, Echeverri F, Mahboubi A, Green DR: DNA damaging agents induce expression of Fas ligand and subsequent apoptosis in T lymphocytes via the activation of NF-κB and AP-1. Mol Cell 1998; 1: 543–51.
9. Prasad AV, Mohan N, Chandrasekar B, Meltz ML: Activation of nuclear factor κB in human lymphoblastoid cells by low-dose ionizing radiation. Radiat Res 1994; 138: 367–72.
10. Mohan N, Meltz ML: Induction of nuclear factor kappa B after low-dose ionizing radiation involves a reactive oxygen intermediate signaling pathway. Radiat Res 1994; 140: 97–104.
11. Tanaka K: Proteasomes: structure and biology. J Biochem 1998; 123: 195–204.
12. Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL: A proteolytic system that compensates for loss of proteasome function. Nature 1998; 392: 618–22.
13. Hildebrandt G, Seed MP, Freemantle CN, Alam CA, Colville-Nash PR, Trott KR: Mechanisms of the anti-inflammatory activity of low-dose radiation therapy. Int J Radiat Biol 1998; 74: 367–78.

Am J Clin Oncol (CCT) 24(5): 481–485, 2001.

© 2001 Lippincott Williams & Wilkins, Inc., Philadelphia

Molecular Pathways That Modify Tumor Radiation Response

Milena Pervan, B.S., Frank Pajonk, M.D., Ph.D., Ji-Rong Sun, M.D., H. Rodney Withers, M.D., D.Sc., and William H. McBride, D.Sc.

Aberrant expression of signal transduction molecules in pathways controlling cell survival, proliferation, death, or differentiation are a common feature of all tumors. The identification of the molecules that are involved allows the development of novel tumor-specific strategies. Not surprisingly, targeting these pathways often also results in radiosensitization. The efficacy of such directed therapies may, however, be limited by the heterogeneity and the multiple mutations that are associated with the cancerous state. A more robust alternative may be to target global mechanisms of cellular control. The ubiquitin/proteasome degradation pathway is one candidate for such therapeutic intervention. This pathway is the main posttranscriptional mechanism that controls levels of many short-lived proteins involved in regulation of cell cycle progression, DNA transcription, DNA repair, and apoptosis. Many of these proteins are involved in various malignancies and/or radiation responses. In recent years, proteasome inhibitors have gained interest as a promising new group of antitumor drugs. PS-341, a reversible inhibitor of proteasome chymotryptic activity, is currently being tested in phase I clinical trials. In this study, we show that proteasome inhibition by PS-341 can alter cellular radiosensitivity in vitro and in vivo, in addition to having direct antitumor effects.

Key Words: Signal transduction pathways—Radiation response—Radiosensitization—Proteasome—PS-341.

In recent years, numerous studies have demonstrated that the cellular response to irradiation can be modified by intracellular or extracellular manipulation of signal transduction pathways.[1–6] Those studies dealing with the effects of cytokines/growth factors, cell–cell contact, and extracellular matrix interactions often reflect classic radiobiologic experiments demonstrating repair of potentially lethal damage. The primary role of these extracellular signals in organized tissues is to act in concert with the programmed expression of specific receptors to define a sense of position and to control cell proliferation and differentiation under physiologic and damage conditions. Mispositioning of a cell leads to homelessness (or "anoikesis") and a tendency toward death by "neglect" because of a lack of appropriate signals. It also tends to result in a state of relative radiosensitivity.[7,8] In vivo, this can be seen in a frequent coincidence of the spatial distribution of apoptotic cells after tissue irradiation with areas of active proliferation.[9] Indeed, radioresponsiveness in general is likely to be spatially dependent and governed by both the signal transduction pathways preexisting in a cell before irradiation, and those activated by irradiation.

The difference between tumors and normal tissues is that the former have mutated genes that affect the signaling pathways that control cell proliferation, differentiation, or death. These mutations allow them to circumvent positional control mechanisms and survive in a state of relative positional independence. Signal transduction pathways become reequilibrated, and the cells become addicted to specific pathways for survival. The therapeutic advantage is that tumor cells are very sensitive to blockade of these pathways and they may serve as an "Achilles heel" for that cancer. Identification and characterization of these pathways in individual cancers is therefore very important for selection of an appropriate specific therapy. New gene discovery techniques will be extremely useful in this regard. Already a number of molecular cell pathways that are mutated in cancer and that appear to determine survival or death after irradiation have been identified, and various agents, primarily designed as sole therapies, have been devised that also serve to radiosensitize.[10] Although optimism for pathway-specific therapies for cancer is high, it should be noted that: (1) overexpression of a known survival factor does not necessarily indicate its involvement in an addictive pathway or characterize a radioresistant tumor;[11] (2) the pathways are, to an extent, cell type-specific; and (3) there is much yet to be learned about the apparently intimate relationship between proliferative/growth arrest and survival/death pathways and radiosensitization.

Because cancers acquire multiple mutations and develop heterogeneity that might prevent a complete cure if

From the Department of Radiation Oncology, Roy E. Coats Research Laboratories and Jonsson Comprehensive Cancer Center, UCLA School of Medicine, Los Angeles, CA, U.S.A.

Dr. Frank Pajonk's current address is Department of Radiation Therapy, Radiological University Clinic Freiburg, Germany. Dr. Ji-Rong Sun's current address is Amgen Corporation, Thousand Oaks, California, U.S.A.

Supported by US Army/Medical Research Acquisition Activity, DAMD 17-00-1-0076.

Address correspondence and reprint requests to Dr. William H. McBride, Department of Radiation Oncology, Roy E. Coats Research Laboratories, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1714, U.S.A.

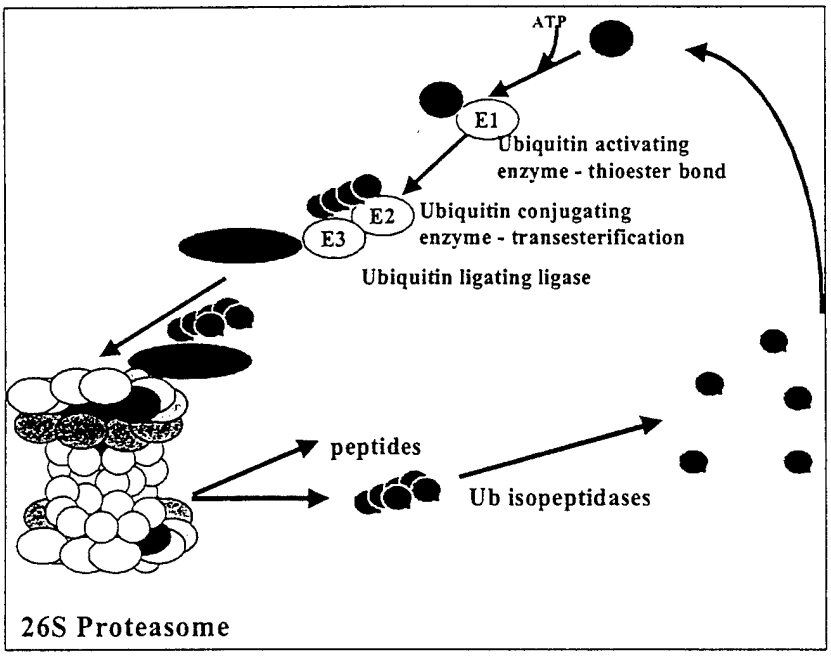


FIG. 1. Ubiquitin/proteasome degradation pathway. Ubiquitin is a 76-residue protein that can be attached by an isopeptide bond between the e-amino group of lysine on the target and the C-terminal glycine of ubiquitin by a series of activating (E1), conjugating (E3), and ligating (E3) enzymes or adaptor molecules. The polyubiquitinated product then becomes the target for 26S proteasome degradation with recycling of ubiquitin by isopeptidases.

single molecules were targeted as a sole therapy, combination of these biologic approaches with cytotoxic therapies, such as radiotherapy, would seem desirable, in particular, because radio- and chemosensitization is a frequent consequence of treatment. In addition, targeting "downstream" nodal points at which multiple pathways converge may be a better therapeutic strategy than targeting a single gene, a single protein, or a single afferent molecular pathway.

One potential new target for cancer therapy that has recently emerged is the ubiquitin/proteasome system (Fig. 1). This pathway is the main posttranscriptional mechanism that simultaneously controls levels of many short-lived proteins involved in regulation of cell cycle progression, DNA transcription, DNA repair, apoptosis, angiogenesis, inflammation, immunity, and cell growth. Some of the proteins regulated by proteasomal degradation that are relevant to radiation include p53,[12–14] mdm2,[15] p21,[16,17] p27,[18] RB,[19] cyclins A, B and E,[20–22] NF-κB/IκB,[23,24] c-Myc,[25–27] c-Jun,[28] c-Fos,[29] HIF-1α,[30,31] DNA-PKcs,[32] rad23,[33] Bcl-2,[34–36] bax,[37] and caspase-3.[38,39] Many of these molecules are radiation inducible through posttranslational, proteasome-dependent mechanisms and modulate cellular responses to irradiation. Indeed, the proteasome itself appears to be a redox-sensitive target for radiation.[40] The ubiquitin/proteasome system promises to yield a large variety of potential anticancer strategies. In the future, many of these may be directed against the E3 ubiquitin ligases that target specific molecules for destruction. The reversible nonspecific inhibitor of proteasome chymotrypsin-like activity, PS-341 is the first agent deliberately targeting this system to enter phase I clinical trials for cancer.[41]

The consequences of inhibiting proteasome activity with reversible and nonreversible drugs have been described in numerous in vitro studies as cell cycle arrest and death by apoptosis.[36,38,42] In addition, we have shown that the reversible proteasome inhibitor MG-132 can radiosensitize cells in vitro.[24] PS-341 has been previously shown to slow tumor growth in vivo,[43] and, in this study, we show that it can affect cellular radiosensitivity in vitro and in vivo.

PROTEASOME INHIBITORS INCREASE RADIOSENSITIVITY OF CANCER CELLS

Our in vitro experiments with proteasome inhibitors MG-132 and PS-341 show synergistic effects of the drugs and ionizing radiation on the survival of TRAMP-C1 mouse prostate carcinoma cells. For these experiments, cells were treated with 100 nmol/l PS-341 and 10 μmol/l MG-132 (50% survival doses) for 3 hours, and the drug was washed out before irradiation. Treatment resulted in dose enhancement ratios of 1.5 and 1.2, respectively (Fig. 2A). To test the dose dependence of the radiosensitization effect, experiments with different doses of PS-341 were performed (Fig. 2B). These revealed that the drug has a radiosensitizing effect at doses as low as 2.5 and 5 nmol/l (dose enhancement ratios of 1.1 and 1.5, respectively), cause only partial inhibition of

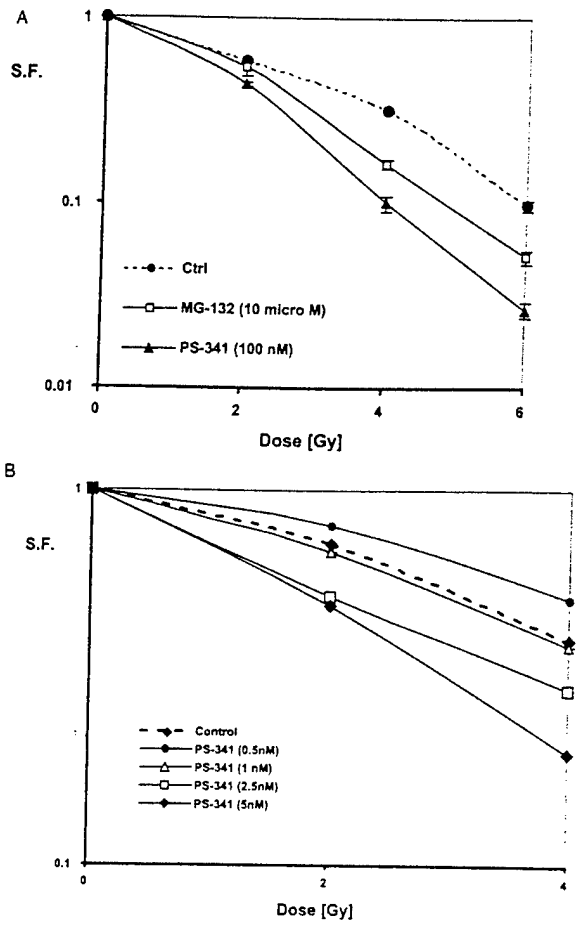


FIG. 2. Clonogenic assay. Murine TRAMP-C1 prostate adenocarcinoma cells were trypsinized, washed, and incubated at 37°C in the presence of (**A**) 100 nmol/l PS-341 or 10 μmol/l MG-132 or (**B**) 0.5, 1, 2.5, and 5 nmol/l PS-341. Three hours later, cells were washed to remove drugs, irradiated with 2, 4, or 6 Gy, and plated in culture dishes. After 14 days cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Survival fraction is expressed as percentage of control.

proteasome activity (30–50%) and are minimally toxic, causing less than 15% cell death. At very low dose levels (<1 nmol/l), PS-341 seems to have no effect on clonogenic cell survival after irradiation, and it may even act to slightly protect cells.

PS-341 has been shown to slow the growth of PC3 tumors in vivo.[43] We observed a similar delay in tumor growth after combined treatment with PS-341, and the effect of irradiation that was at least additive over drug or radiation treatment alone (Fig. 3). Mice treated with 0.3 mg/kg of PS-341 intravenously 3 hours before a single dose of 25-Gy radiation showed a 15-day tumor growth delay versus 6 to 7 days with radiation or drug alone.

DISCUSSION

Targeting individual molecules that are aberrantly expressed in specific cancers can often lead to tumor radiosensitization. This approach has enormous appeal, but may be limited by the heterogeneity and multiple mutations that are associated with the cancerous state. Targeting downstream nodal points involving multiple pathways may be a more robust strategy. Perhaps the ultimate downstream pathway for molecules involved in radiation responses is the proteasome. The proteasome inhibitor PS-341 has entered phase I clinical trials alone and in combination with chemotherapeutic agents (fluorouracil and leucovorin)[41] (Adams J, personal communication, 2000). It appears to be surprisingly well tolerated in mice and humans, provided the intertreatment interval is sufficient to protect against gastrointestinal toxicity.[42] Proteasome inhibitors have been shown to result in apoptosis of almost all cancer cells by mechanisms that have yet to be fully elucidated. Drexler,[44] as well as Soldatenkov and Dritschilo,[45] has suggested that activation of a Bcl-2-sensitive pathway is involved, whereas Hermann and coworkers[46] excluded Bcl-2 and p53 involvement. Normal cells, in contrast, appear to be less affected by treatment, and may even be initially rescued from apoptosis induced by ionizing radiation, glucocorticoids, or phorbol ester inhibitors.[47] Cycling cells are perhaps more sensitive than nonproliferating cells because of the dependency of the cyclin/cdk system on proteasome function.[48] It is also possible that the addiction of cancer cells for various stress proteins gives them greater sensitivity to proteasome inhibition. For example, NF-κB is a survival pathway that is overexpressed in numerous cancers, and activation of NF-κB is tightly controlled through degradation of its inhibitor IκB by the ubiquitin/proteasome system. Proteasome inhibition results in inhibition of NF-κB expression, and, although this does not necessarily result in radiosensitization,[11] cells that are addicted to this pathway may be particularly sensitive. This may explain the promising preliminary responses to PS-341 treatment seen in multiple myeloma (Adams J, personal communication, 2000), where NF-κB expression is probably maintained through the action of interleukin-6 acting in as an autocrine growth factor.

The importance of the ubiquitin/proteasome system for cellular function and responses to therapy has only recently come to the fore. This system integrates with phosphorylation/dephosphorylation as a master controller of intracellular events and is assuming an importance at least as great as these better understood pathways. Expression of many of the molecules critically involved in DNA repair, cell death, and stress reactions are modulated through this pathway. It is redox and radiation sensitive and has proved to be intimately involved in radiation responses at many different levels.[40] Although the extent of the diversity of proteasome structures and ubiquitin-related molecules has yet to be fully appreciated, and their relevance to therapeutic responses yet to

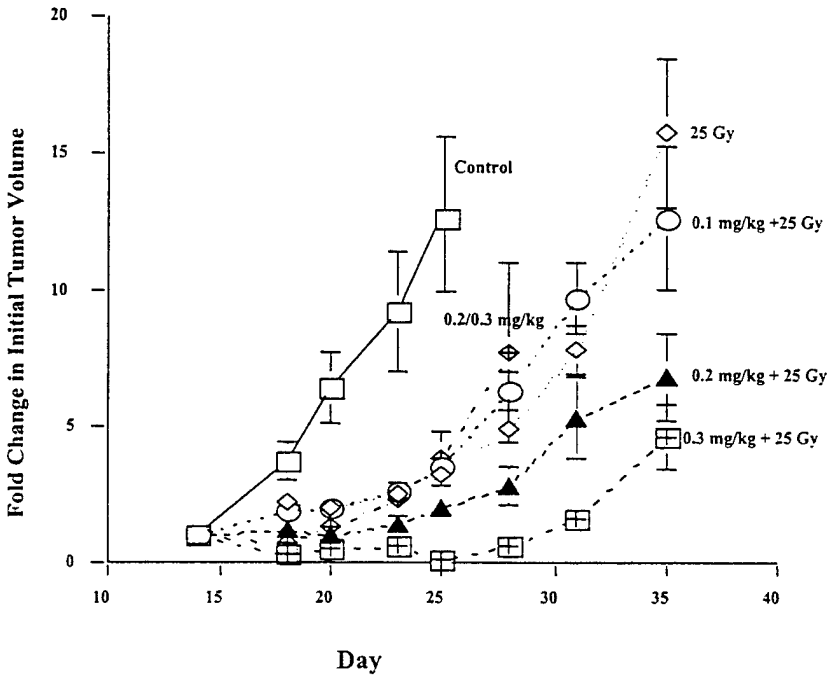


FIG. 3. In vivo tumor growth assay. TRAMP-C1 cells (5×10^5) were implanted subcutaneously in the hind legs of 8-week-old C57/Bl6 mice. When tumors were 6 mm in diameter, animals were treated with different doses of PS-341 (0.1, 0.2, and 0.3 mg/kg intravenously) alone or in combination with 25-Gy local tumor irradiation. Tumor volume was measured until tumors reached 12 mm in diameter.

be elucidated, this system promises to yield strategically important targets for cancer treatment, including improving the therapeutic benefit of radiation therapy. ₡

Acknowledgment: The authors thank Dr. J. Adams (Millennium Pharmaceuticals) for provision of PS-341 and discussion.

REFERENCES

1. Yarnold J. Molecular aspects of cellular responses to radiotherapy. *Radiother Oncol* 1997;44:1–7.
2. Szumiel I. Monitoring and signaling of radiation-induced damage in mammalian cells. *Radiat Res* 1998;150:S92–101.
3. Schmidt-Ullrich RK, Dent P, Grant S, et al. Signal transduction and cellular radiation responses. *Radiat Res* 2000;153:245–57.
4. Sartor CI. Biological modifiers as potential radiosensitizers: targeting the epidermal growth factor receptor family. *Semin Oncol* 2000;27:15–20; discussion 92–100.
5. Zaugg K, Rocha S, Resch H, et al. Differential p53-dependent mechanism of radiosensitization in vitro and in vivo by the protein kinase C-specific inhibitor PKC412. *Cancer Res* 2001;61:732–8.
6. Aebersold DM, Kollar A, Beer KT, et al. Involvement of the hepatocyte growth factor/scatter factor receptor c- met and of Bcl-xL in the resistance of oropharyngeal cancer to ionizing radiation. *Int J Cancer* 2001;96:41–54.
7. Kwok TT, Sutherland RM. The influence of cell-cell contact on radiosensitivity of human squamous carcinoma cells. *Radiat Res* 1991;126:52–7.
8. Moussa H, Mitchell SA, Grenman R, et al. Cell-cell contact increases radioresistance in head and neck carcinoma cell lines. *Int J Radiat Biol* 2000;76:1245–53.
9. Potten CS, Merritt A, Hickman J, et al. Characterization of radiation-induced apoptosis in the small intestine and its biological implications. *Int J Radiat Biol* 1994;65:71–8.
10. Ruifrok AC, McBride WH. Growth factors: biological and clinical aspects. *Int J Radiat Oncol Biol Phys* 1999;43:877–81.
11. Pajonk F, Pajonk K, McBride WH. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells [see comments]. *J Natl Cancer Inst* 1999;91:1956–60.
12. Maki CG, Huibregtse JM, Howley PM. In vivo ubiquitination and proteasome-mediated degradation of p53. *Cancer Res* 1996;56:2649–54.
13. Scheffner M. Ubiquitin, E6-AP, and their role in p53 inactivation. *Pharmacol Ther* 1998;78:129–39.
14. Kubbutat MH, Jones SN, Vousden KH. Regulation of p53 stability by Mdm2. *Nature* 1997;387:299–303.
15. Fang S, Jensen JP, Ludwig RL, et al. Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* 2000;275:8945–51.
16. Blagosklonny MV, Wu GS, Omura S, et al. Proteasome-dependent regulation of p21WAF1/CIP1 expression. *Biochem Biophys Res Commun* 1996;227:564–9.
17. Kibbe MR, Nie S, Seol DW, et al. Nitric oxide prevents p21 degradation with the ubiquitin-proteasome pathway in vascular smooth muscle cells. *J Vasc Surg* 2000;31:364–74.
18. Chiarle R, Budel LM, Skolnik J, et al. Increased proteasome degradation of cyclin-dependent kinase inhibitor p27 is associated with a decreased overall survival in mantle cell lymphoma. *Blood* 2000;95:619–26.
19. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* 1996;56:4620–4.
20. Aristarkhov A, Eytan E, Moghe A, et al. E2-C, a cyclin-selective ubiquitin carrier protein required for the destruction of mitotic cyclins. *Proc Natl Acad Sci USA* 1996;93:4294–9.
21. Hershko A, Ganoth D, Pehrson J, et al. Methylated ubiquitin

inhibits cyclin degradation in clam embryo extracts. *J Biol Chem* 1991;266:16376–9.
22. King RW, Peters JM, Tugendreich S, et al. A 20S complex containing CDC27 and CDC16 catalyzes the mitosis-specific conjugation of ubiquitin to cyclin B. *Cell* 1995;81:279–88.
23. Alkalay I, Yaron A, Hatzubai A, et al. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 1995;92:10599–603.
24. Pajonk F, Pajonk K, McBride WH. Apoptosis and radiosensitization of Hodgkin cells by proteasome inhibition. *Int J Radiat Oncol Biol Phys* 2000;47:1025–32.
25. Flinn EM, Busch CM, Wright AP. myc boxes, which are conserved in myc family proteins, are signals for protein degradation via the proteasome. *Mol Cell Biol* 1998;18:5961–9.
26. Gregory MA, Hann SR. c-Myc proteolysis by the ubiquitin-proteasome pathway: stabilization of c-Myc in Burkitt's lymphoma cells. *Mol Cell Biol* 2000;20:2423–35.
27. Bahram F, von der Lehr N, Cetinkaya C, et al. c-Myc hot spot mutations in lymphomas result in inefficient ubiquitination and decreased proteasome-mediated turnover. *Blood* 2000;95:2104–10.
28. Hermida-Matsumoto ML, Chock PB, Curran T, et al. Ubiquitinylation of transcription factors c-Jun and c-Fos using reconstituted ubiquitinylating enzymes. *J Biol Chem* 1996;271:4930–6.
29. Tsurumi C, Ishida N, Tamura T, et al. Degradation of c-Fos by the 26S proteasome is accelerated by c-Jun and multiple protein kinases. *Mol Cell Biol* 1995;15:5682–7.
30. Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* 1997;272:22642–7.
31. Huang LE, Gu J, Schau M, et al. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* 1998;95:7987–92.
32. Wu LW, Reid S, Ritchie A, et al. The proteasome regulates caspase-dependent and caspase-independent protease cascades during apoptosis of MO7e hematopoietic progenitor cells. *Blood Cells Mol Dis* 1999;25:20–9.
33. Schauber C, Chen L, Tongaonkar P, et al. Rad23 links DNA repair to the ubiquitin/proteasome pathway. *Nature* 1998;391:715–8.
34. Dimmeler S, Breitschopf K, Haendeler J, et al. Dephosphorylation targets Bcl-2 for ubiquitin-dependent degradation: a link between the apoptosome and the proteasome pathway. *J Exp Med* 1999; 189:1815–22.
35. Chadebech P, Brichese L, Baldin V, et al. Phosphorylation and proteasome-dependent degradation of Bcl-2 in mitotic-arrested cells after microtubule damage. *Biochem Biophys Res Commun* 1999;262:823–7.
36. Marshansky V, Wang X, Bertrand R, et al. Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. *J Immunol* 2001;166:3130–42.
37. Li B, Dou QP. Bax degradation by the ubiquitin/proteasome-dependent pathway: involvement in tumor survival and progression. *Proc Natl Acad Sci USA* 2000;97:3850–5.
38. Pasquini LA, Besio Moreno M, Adamo AM, et al. Lactacystin, a specific inhibitor of the proteasome, induces apoptosis and activates caspase-3 in cultured cerebellar granule cells. *J Neurosci Res* 2000;59:601–11.
39. Qiu JH, Asai A, Chi S, et al. Proteasome inhibitors induce cytochrome c-caspase-3-like protease-mediated apoptosis in cultured cortical neurons. *J Neurosci* 2000;20:259–65.
40. Pajonk F, McBride WH. Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. *Radiother Oncol* 2001;59:203–12.
41. Wright J, Hillsamer VL, Gore-Langton RE, et al. Clinical trials referral resource. Current clinical trials for the proteasome inhibitor PS-341. *Oncology* 2000;14:1589–90, 1593–4, 1597.
42. Adams J, Palombella VJ, Elliott PJ. Proteasome inhibition: a new strategy in cancer treatment. *Invest New Drugs* 2000;18:109–21.
43. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. *Cancer Res* 1999;59:2615–22.
44. Drexler HC. Activation of the cell death program by inhibition of proteasome function. *Proc Natl Acad Sci USA* 1997;94:855–60.
45. Soldatenkov VA, Dritschilo A. Apoptosis of Ewing's sarcoma cells is accompanied by accumulation of ubiquitinated proteins. *Cancer Res* 1997;57:3881–5.
46. Herrmann JL, Briones F Jr, Brisbay S, et al. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. *Oncogene* 1998;17: 2889–99.
47. Grimm LM, Goldberg AL, Poirier GG, et al. Proteasomes play an essential role in thymocyte apoptosis. *EMBO J* 1996;15:3835–44.
48. Machiels BM, Henfling ME, Gerards WL, et al. Detailed analysis of cell cycle kinetics upon proteasome inhibition. *Cytometry* 1997; 28:243–52.

Int. J. Radiation Oncology Biol. Phys., Vol. 47, No. 4, pp. 1025–1032, 2000
Copyright © 2000 Elsevier Science Inc.
Printed in the USA. All rights reserved
0360-3016/00/$–see front matter

ELSEVIER

PII S0360-3016(00)00516-2

BIOLOGY CONTRIBUTION

APOPTOSIS AND RADIOSENSITIZATION OF HODGKIN CELLS BY PROTEASOME INHIBITION

FRANK PAJONK, M.D.,[1] KATJA PAJONK,[1] AND WILLIAM H. MCBRIDE, D.SC.

Department of Radiation Oncology, Experimental Division, Roy E. Coats Research Laboratories, UCLA School of Medicine, Los Angeles, CA

Purpose: Malignant cells from Hodgkin's disease have been reported to be defective in regulation of NF-κB activity. Ionizing radiation is known to activate NF-κB, and it has been suggested that this pathway may protect cells from apoptosis following exposure to radiation and other therapeutic agents. Defective NF-κB regulation in Hodgkin cells could therefore dictate the response of this disease to therapy, as well as be responsible for maintaining the malignant phenotype. The purpose of this study was to explore whether NF-κB activity could be modulated in Hodgkin cells and whether it determines the response of these cells to treatment with ionizing radiation and/or dexamethasone.

Methods and Materials: Activation of NF-κB in cells is accomplished in large part by degradation of its inhibitor IκB through the 26s proteasome. HD-My-Z Hodgkin cells were treated with the proteasome inhibitor MG-132 or transduced with a dominant negative super-repressor IκBα. Clonogenic survival, apoptosis, proteasome activity, and NF-κB binding activity were monitored in response to ionizing radiation and/or dexamethasone treatment.

Results: HD-My-Z Hodgkin cells had modest NF-κB levels but, unlike other cell types, did not decrease their level of constitutively active NF-κB in response to proteasome inhibition with MG-132. In contrast, transduction with a non-phosphorable IκBα construct abolished expression. MG-132 did, however, induce apoptosis in HD-My-Z cells and sensitized them to ionizing radiation. Dexamethasone treatment had no effect on NF-κB activity or clonogenic survival of Hodgkin cells, but protected them from irradiation.

Conclusion: We conclude that inhibition of 26s proteasome activity can induce apoptosis in HD-My-Z Hodgkin cells and radiosensitize them, in spite of the fact that their constitutively active NF-κB levels are unaltered. The proteasome may be a promising new therapeutic target for intervention in this disease. In contrast, the use of glucocorticoids in conjunction with radiation treatment for this tumor may require re-evaluation. © 2000 Elsevier Science Inc.

Hodgkin's disease, 26s Proteasome, NF-κB, MG-132, Dexamethasone.

INTRODUCTION

The treatment of Hodgkin's disease has improved greatly over the last three decades. In the last 10 years, stage-adapted radio/chemotherapy protocols have been introduced that reduce overall treatment failure to less than 25 % (1–5). Even salvage therapies of relapsed Hodgkin's disease are highly effective, with long-term remission rates of more than 50% (6–8). In spite of this success, there are a number of patients with primary or relapsed Hodgkin's disease whose tumors are resistant to both radiotherapy and multi-agent chemotherapy. Others with relapsed disease cannot be treated because of hematological or local complications caused by prior treatment. New treatment modalities, preferably based on insights into the pathophysiology of Hodgkin's disease, are needed for these patients.

The malignant cells in Hodgkin's disease, the Hodgkin cell and the multinuclear Reed-Sternberg cell have recently been shown to belong to the B-lymphocyte lineage (9–11). However, in contrast to most B-lymphocytes. Hodgkin's lymphoma cells appear relatively resistant to apoptosis. Recently, Bargou and coworkers (12) observed a feature of Hodgkin cell lines that might explain this resistance. All Hodgkin cell lines tested showed constitutive activation of nuclear factor kappa B (NF-κB). Activation of NF-κB is known to protect many cell types from apoptotic death (13–15).

NF-κB is a hetero- or homodimer of proteins that recognize a specific DNA-binding motif in the promoter region of many genes, especially those involved in inflammatory responses. In its inactive form, the binding region (NLS) is

Reprint requests to: Dr. William McBride, Department of Radiation Oncology, Roy E. Coats Research Laboratories, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1714. Tel.: (310) 825-8589; Fax: (310) 206-1260; E-mail: mcbride@radonc.ucla.edu

Acknowledgments—We are grateful to Dr. Heike Pahl (Department of Experimental Anesthesia, University Clinic Freiburg, Germany) for technical advise in gel-shift experiments and Dr. R. Batra (VA Hospital, Los Angeles, CA) for providing the adenoviral vectors.

Accepted for publication 5 January 2000.

[1]These authors contributed equally to this study.

covered by inhibitor proteins called IκB. NF-κB activation most often results from phosphorylation of IκB by IκB kinases (Iκ), its ubiquitination, and subsequent degradation by the multicatalytic 26s proteasome. This degradation enables nuclear translocation of NF-κB and initiation of gene expression (16, 17). While many events could cause constitutive activation of NF-κB, there are reports that Hodgkin cell lines may have mutated IκBα, resulting in a nonfunctional IκBα protein (18) or an almost complete lack of IκBα gene expression (19). Although only a few cell lines are available for study, it is possible that unregulated expression of NF-κB is responsible for the pathogenesis of this disease. In this study, we explored the status of NF-κB in the HD-My-Z Hodgkin cell line, the mechanism underlying its constitutive expression, and whether abnormalities in this pathway affect cell death following exposure to proteasome inhibitors and/or ionizing irradiation and steroids.

METHODS AND MATERIALS

Cell culture

The Hodgkin cell line HD-My-Z (DSMZ, Braunschweig) has been described in detail elsewhere (19). This, as well as PC3 and LnCaP human prostate carcinoma (ATCC), SW 1088 astrocytoma (ATCC), ECV 304 bladder carcinoma cells, and A549 non-small cell lung cancer (ATCC) cell line, were grown in 75-cm^2 flasks (Falcon) at 37°C in a humidified atmosphere at 5% CO_2. The medium was DMEM medium (Gibco/BRL) supplemented with 10% FCS (Sigma), 1% penicillin/streptomycin (Sigma), and 0.5 g/mL fungizone (amphotericin B, Gibco/BRL). For studies of proteasome and NF-κB functional activity, 10^6 cells were plated into 10-cm culture dishes 24 h before the start of the experiment.

Cell extracts and electrophoretic mobility shift assays

For preparation of total cellular extracts, cells were mechanically scraped from the plate, washed with ice-cold PBS, and lysed in TOTEX-buffer (20 mM HEPES [pH 7.9], 0.35 mM NaCl, 20% glycerol, 1% NP-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM DTT, PMSF, and aprotinin for 30 min on ice). Lysate was centrifuged at 12,000 *g* for 5 min. Protein concentration was determined using the BCA protocol (Pierce) with bovine albumin as standard. Fifteen micrograms of protein of the resulting supernate were incubated for 25 min at room temperature with 2 μl BSA (10 μg/μl), 2 μl dIdC (1 μg/μl), 4 μl Ficoll-buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, 0.1 mM PMSF), 2 μl buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1 mM PMSF) and 1 μl of the [γ^{32}P] ATP-labeled oligonucleotide (Promega, NF-κB: AGT TGA GGG GAC TTT CCC AGG). For a negative control, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5% TBE gels. Dried gels were placed on a phosphor screen for 24 h and analyzed on a phosphor imager (Storm 860, Molecular Dynamics).

Irradiation

HD-My-Z cells, which grow as a loosely adherent monolayer, were dislodged by shaking, counted, and diluted to a concentration of 10^6 cells/mL. The cell suspension was immediately irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shephard, Mark I) at a dose rate of 580 rad/min. Corresponding controls were sham irradiated.

Clonogenic survival

Colony-forming assays were performed immediately after irradiation by plating an appropriate number of cells (2×10^3–2×10^4) into Petri dishes, in triplicate. After 14 days' culture, cells were fixed, stained with crystal violet, and colonies consisting of more than 50 cells were counted. Data shown resulted from a minimum of three independent experiments.

Proteasome function assays

Proteasome function was measured as described previously (20). Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM MgCl2, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl^2$, 2 mM ATP, 250 mM sucrose) were added and vortexed for 1 min. Beads and cell debris were removed by centrifugation at 1,000 *g* for 5 min and 10,000 *g* for 20 min. Protein concentration was determined by the BCA protocol (Pierce). Ten micrograms of protein of each sample was diluted with buffer I to a final volume of 100 μl. The fluorogenic proteasome substrate SucLLVY-7-amido-4-methylcoumarin (chymotrypsin-like, Sigma) and the proteasome inhibitor MG-132 (Calbiochem) were dissolved in DMSO and added in a final concentration of 100 μM in 1% DMSO. Controls received diluent only. Samples were incubated for 45 min at 37°C. The reaction was stopped by the addition of 1 mL SDS (1%) and free 7-amido-4-methylcoumarin was determined using a fluorescence plate reader (fmax, Molecular Devices) at 380/460 nm.

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodiumvanadate). Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin as standard. One hundred micrograms of protein were subjected to SDS gel electrophoresis (0.1% SDS in Tris-HCl/10% polyacrylamide, pH 8.8 and a 4% polyacrylamide stacking gel, pH 6.8) and blotted overnight to PVDF membranes (4°C). After blocking with Blotto-buffer (PBS, 2% skim milk) membranes were incubated with a polyclonal antibody against human IκBα (Santa Cruz Biotechnologies). A secondary HRP-conjugated antibody and DAB (Sigma) were used for visualization.

Transduction experiments

The recombinant replication-deficient adenoviruses Ad5-IκB and Ad5-LacZ were kindly provided by Dr. R. Batra

(VA Hospital, Los Angeles, CA) and are described elsewhere (21). Ad5-IκB contains a gene for a NF-κB superrepressor IκBα under control of a CMV-promoter/enhancer. The encoded protein contains serine-to-alanine mutations in residues 32 and 36, preventing its phosphorylation, ubiquitination, and subsequent degradation by the proteasome. The Ad5-LacZ is a control virus that contains the gene for β-galactosidase in place of IκBα. Cells were plated into Petri dishes (10 cm, Falcon). After 24 h, the medium was changed and viral vectors containing non-phosphorable IκBα or β-galactosidase genes were added at different multiplicities of infection (MOIs). After a 2-h incubation, the virus-containing medium was replaced by fresh medium and cells were incubated for an additional 48 h to allow gene expression.

Determination of apoptosis

Apoptotic cells were detected using the *In Situ* Cell Death Kit (Boehringer Mannheim). The manufacturer's protocol was followed with some minor modifications. Briefly, attached and detached cells were collected, centrifuged, fixed with 100% ethanol, washed with PBS, and pelleted by centrifugation for 5 min at 2,000 *g*. Cells were permeabilized by resuspension in 0.1% Triton X-100 in 0.1% sodium citrate and incubation for 2 min on ice. Cells were washed twice in PBS, resuspended in TUNEL reaction mixture, and incubated for 60 min at 37°C. After three washes with PBS, fluorescence was measured at 518 nm using a flow cytometer (FACScan, Becton Dickinson) and analyzed using the CellQuest software (Becton Dickinson).

Cell cycle analysis

A sample consisting of 5×10^5 cells per sample were washed, fixed with 70% ethanol, and stained by propidium iodide (0.1 mg/mL), and 0.1% Nonidet NP-40. After treatment with RNAse (1 mg/mL), the cell cycle parameters were determined from the DNA content using a flow cytometer (FACScan).

Statistics

For statistical analysis of the survival plots, the data was fitted using a generalized linear model and the statistical software package JMP (version 3.2 for Macintosh Computers, SAS). Two curves were assumed to be significantly different from each other if F-distribution values showed significance at a *p*-value of 0.05.

RESULTS

NF-κB activity in HD-My-Z Hodgkin cells

It has been suggested that Hodgkin cells have high constitutive NF-κB levels (12, 22). To test this in HD-My-Z cells, gel shift assays for functionally active NF-κB were performed on cell extracts and the results compared with those for other cancer cell lines (A549, ECV 304, LnCaP, PC3, and SW1088). Surprisingly, constitutive NF-κB levels in HD-My-Z cells were lower than those in most of the other cell lines. For example, NF-κB DNA-binding activity in extracts from PC3 cells was almost three times higher than that in HD-My-Z cells (Fig.1: lanes 2, 4, 6, 8, 10, and 12).

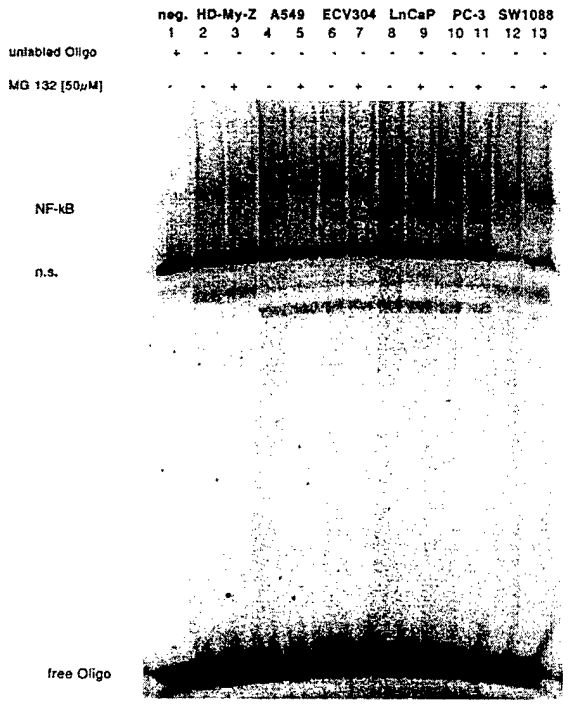


Fig. 1. The electrophoretic mobility shift assay shows constitutive NF-κB DNA-binding activity in cytosolic protein extracts (15 μg) from six different human tumor cell lines: HD-My-Z (lane 2), A549 (lane 4), ECV304 (lane 6), LnCaP (lane 8), PC3 (lane 10), and SW 1088 (lane 12) 24 h after plating. To show specificity of the binding reaction, a 50-fold molar excess of the unlabeled consensus oligonucleotide was added in lane 1. Preincubation of the cells for 3 h with the reversible proteasome inhibitor MG-132 (50 μM) decreased constitutive NF-κB binding activity in all cell lines except HD-My-Z (lanes 3, 5, 7, 9, 11, and 13).

Inhibition of the 26s proteasome does not decrease NF-κB activity in HD-My-Z cells

NF-κB activation is normally controlled by degradation of its inhibitor, IκB, through the proteasome. Blocking proteasome activity using the specific inhibitor MG-132 should therefore lead to a decrease in NF-κB activity. This was found to be the case for A549, ECV 304, LnCaP, PC3, and SW1088 cells treated for 3 h with 50 μM MG-132 (Fig. 1: lanes 5, 7, 9, 11, and 13). In contrast, the same treatment did not affect NF-κB activity in HD-My-Z cells (lane 3), indicating that constitutive NF-κB activity in this cell line is not regulated by this mechanism.

Clonogenicity of HD-My-Z cells is reduced by expression of a dominant negative IκBα

Because NF-κB activity was not decreased by MG-132 treatment of HD-My-Z cells, its ability to be modulated by

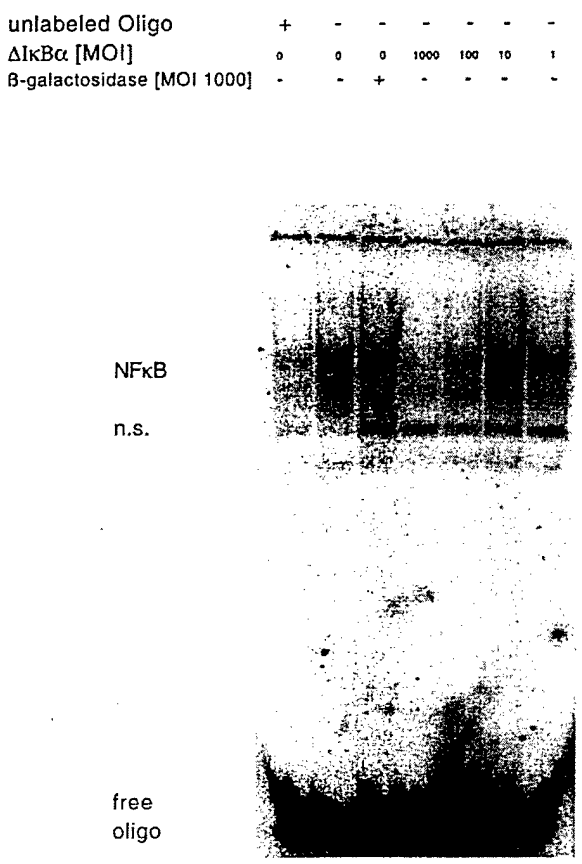


Fig. 2. Gel-shift experiments with extract of HD-My-Z Hodgkin cells after transduction with adenoviral vector carrying a truncated IκBα gene. An MOI of 1,000—equivalent to more than 99.9% cell transduction efficiency—resulted in a complete loss of NF-κB binding activity. Transduction with a β-galactosidase (MOI 1,000) gene did not change NF-κB activity.

IκB was tested by introducing a dominant negative IκBα, which does not undergo degradation through the proteasome. There was an MOI-dependent decrease in NF-κB activation 48 h after transduction (Fig. 2), consistent with regulation by the introduced IκBα. Clonogenicity of the HD-My-Z cells also was decreased by expression of dominant negative IκBα in a dose-dependent manner (Fig. 3). The control vector containing a β-galactosidase gene did not alter plating efficiency.

Proteasome activity is normal in HD-My-Z cells

The above experiments showed that HD-My-Z cells are unable to regulate NF-κB expression through the IκB degradation pathway. To exclude the proteasome as the defect, chymotrypsin-like cleavage activity was measured by fluorogenic assay in HD-My-Z cells and the result was compared with activity in other cancer cell lines. In general, proteasome activity correlated with NF-κB activity for the six cell lines tested (r = 0.863). PC3 cells had the highest activity; SW1088 cells had the lowest. The other cell lines, including HD-My-Z, were in-between these two extremes

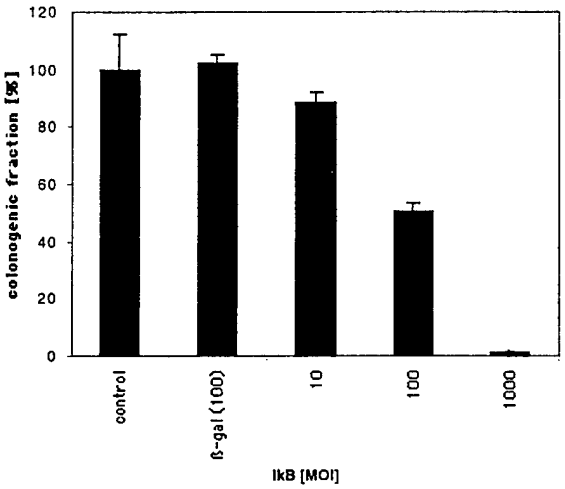


Fig. 3. Decrease in clonogenic fraction of HD-My-Z Hodgkin cells, assayed by measurements of plating efficiency, after expression of a dominant negative IκB. The decrease in clonogenicity in HD-My-Z cells was correlated to the number of viral particles used. The control vector, containing a β-galactosidase (MOI 100) gene, did not affect clonogenicity.

(Fig. 4). Extracts from HD-My-Z cells that were incubated with different concentrations of MG-132 showed dose-dependent inhibition of cleavage activity; 0.25 μM MG-132 inhibited to 50% after a 45-min incubation (Fig. 5). These experiments show that failure of MG-132 to affect NF-κB levels in HD-My-Z cells cannot be attributed to an inability of the drug to block proteasome activity, which appears normal in this cell line.

Further attempts were made to alter NF-κB activity in HD-My-Z cells using MG-132. Three hours' preincubation of cells with 3–50 μM MG-132 had no effect (Fig. 6a), nor did the continuous presence of 50 μM MG-132 over a period of 48 hours' cells (Fig. 6b). Twenty-four and 48 h

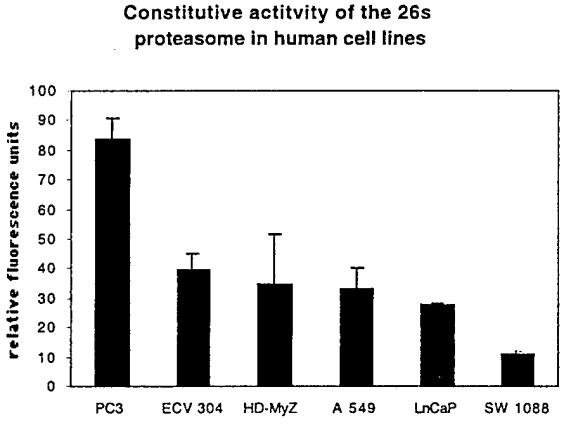


Fig. 4. Constitutive (chymotrypsin-like) proteolytic activity of the 26s proteasome in six different human cell lines. Measurements are expressed in relative fluorescence units and reflect the release of 7-amido-4-methylcoumarin (AMC) from the fluorogenic proteasomal substrate SucLLVY-AMC into the buffer (excitation 380 nm, emission 460 nm).

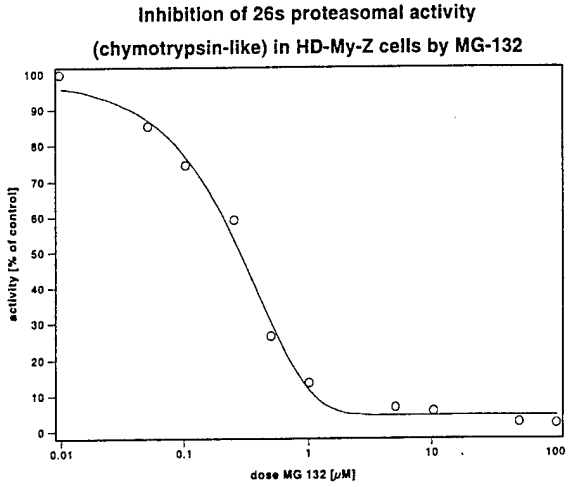


Fig. 5. Inhibitory effect of different concentrations (0, 0.05, 0.1, 0.25, 0.5, 1.0, 5.0, 10, 50, 100 μM) of the specific 26s proteasome inhibitor MG-132 on chymotrypsin-like cleavage activity in cell free extracts of HD-My-Z Hodgkin cells. Release of AMC from the fluorogenic peptide SucLLVY-AMC to the buffer was measured (excitation 380 nm, emission 460 nm) and expressed in percent of cleavage activity of extracts from untreated control cells.

after the start of drug treatment, there was a decrease in NF-κB, but this was associated with cell death. By 24 h, a pro-G_1 population (42%) was observed by flow cytometry that was almost absent (7%) in the control.

Inhibition of proteasome activity in HD-My-Z cells induces apoptosis

The ability of MG-132 treatment to cause death of HD-My-Z cells in the absence of any effect on NF-κB activity was explored further. Three hours' incubation in MG-132 (0–50 μM), followed by washing and incubation for an additional 24 h, caused dose-dependent apoptosis, as assessed by a TUNEL assay (Fig. 7a). The continuous presence of MG-132 in concentrations as low as 31.25 nM, which corresponds to a 10% inhibition of proteasome cleavage activity in cell-free protein extracts, reduced clonogenicity to 40% when compared to the untreated control (Fig 7b). The general correspondence between the dose of MG-132 required to cause cell death and the dose required for inhibition of 26s proteasome activity argue in favor of these processes being mechanistically linked.

Inhibition of proteasome activity in HD-My-Z cells sensitizes them to radiation

Because MG-132 induced apoptosis in HD-My-Z cells without affecting constitutive NF-κB activity, its effect on their response to radiation was examined. HD-My-Z cells were moderately radiation resistant, with a S.F. 2 Gy of 0.56 ± 0.032.(Fig. 8) They were 1.4 times more radioresistant than PC3 cells (S.F. 2 Gy = 0.39 ± 0.026, data not shown). Transient inhibition of proteasome function by 3 hours' preincubation of HD-My-Z cells in 50 μM MG-132 significantly sensitized them to ionizing irradiation, as measured by clonogenic survival (Fig. 8: S.F. 2 Gy = 0.42 ± 0.064, $p < 0.0001$, F-test). MTT assays performed 5 days after short-term incubation (3 h) of HD-My-Z cells confirmed the radiosensitizing effect of MG-132 with lower (1 and 10 μM) doses (data not shown).

In contrast to MG-132, exposure of HD-My-Z cells to dexamethasone (100 μM) for 3 h protected them from irradiation (Fig. 8: S.F. 2 Gy = 0.58 ± 0.042, $p < 0.0001$, F-test; see figure legend for radiobiological parameters). Glucocorticoids are a standard treatment for Hodgkin's disease. They also have been reported to decrease NF-κB in a number of cell lines by increasing IκB levels (23, 24). In this study, NF-κB levels did not decrease in HD-My-Z, PC3, or A459 cells under the conditions of treatment (data not shown). The plating efficiency in HD-My-Z cells was little changed, from 17.2% to 19.8%, by dexamethasone exposure, and there was no apoptosis (data not shown).

DISCUSSION

The malignant cell in Hodgkin's disease is most frequently reported to belong to the B-cell lineage (9–11). In general, B-cells are prone to undergo apoptosis. This is counteracted during immune activation by NF-κB-dependent pathways. It is therefore not surprising that Hodgkin and Reed-Sternberg cells generally show levels of NF-κB DNA-binding activity comparable to activated B-cells (22). In activated B-cells, NF-κB activation depends on CD40-ligand engagement (26). In Hodgkin cells, the factors that maintain NF-κB activity are less clear (18, 26–28).

Constitutive NF-κB activity is not a characteristic restricted to Hodgkin cells. In this study, extracts from other tumor cell lines had constitutive levels of active NF-κB, nor is the level of constitutive activity necessarily high in Hodgkin cells, as has been suggested (12, 22). The HD-My-Z Hodgkin cell line showed lower constitutive activity than most cell lines tested. What was unusual about the HD-My-Z Hodgkin cell line compared to the other cell lines was that the NF-κB activity was not down-regulated by treatment with MG-132, a reasonably specific proteasome inhibitor. It was, however, down-regulated by overexpression of a dominant negative IκB. Proteasome activity in HD-My-Z cells was inhibitable by MG-132 and was in the mid-range compared with the tumor cell lines that were tested. These findings indicate that HD-My-Z cells are most likely defective in IκB. Other Hodgkin cell lines have been reported to have low IκBα mRNA and protein levels (22) that appear to be non-functional (18). Although the HD-My-Z cell line has been reported to lack IκBα expression (19), Western blot analysis of extracts of the HD-My-Z cells detected a specific 38-kDa band for IκBα and degradation of IκBα was blocked by treatment with MG-132 (50 μM) for 3 h (data not shown). It remains to be investigated whether the IκBα is mutated, leading to a nonfunctional protein that is specifically unable to inhibit NF-κB.

Although treatment with MG-132 did not down-regulate NF-κB activity in HD-My-Z cells, it could induce apoptosis and decrease clonogenicity. The mechanism underlying

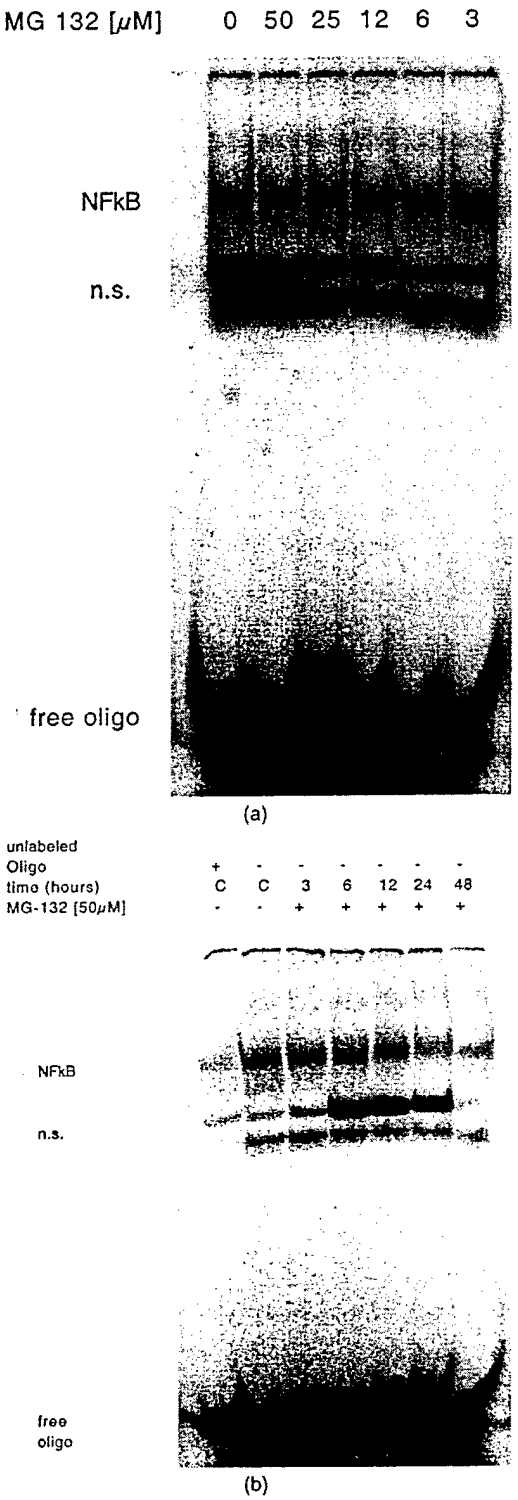


Fig. 6. (a) Gel-shift experiment with cytosolic protein extracts (15 μg) from HD-My-Z cells after 3-h preincubation with MG-132 (50, 25, 12.5, 6.25, and 3.125 μM). Constitutive high NF-κB activity was not affected by inhibition of proteasome function. (b) Incubation of HD-My-Z cells with MG-132 (50 μM) over a time period of 48 h did not change the NF-κB DNA-binding activity during the first 12 h. NF-κB activity decreased only when cells started to die, 24–48 h after start of incubation.

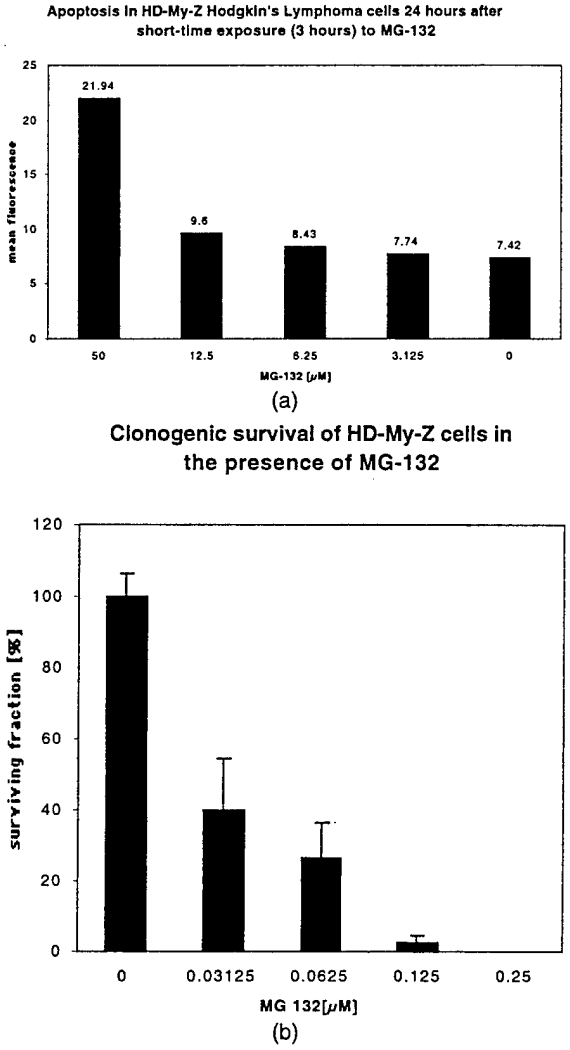


Fig. 7. (a) Apoptosis in HD-My-Z Hodgkin's lymphoma cells 24 h after transient inhibition (3 h) of proteasome function assessed by a TUNEL-assay. MG-132 increased the mean fluorescence signal in a dose-dependent manner. (b) Clonogenicity of HD-My-Z Hodgkin cells, assayed by measurements of the plating efficiency, was drastically decreased by the continuous presence of even low concentrations of MG-132.

these effects remains unclear. It is unlikely to be due to inhibition of proteasome degradation of p53, because HD-My-Z cells constitutively express p53 mRNA and are probably mutated in the p53 gene (19). Recent studies by Herrmann and coworkers excluded any involvement of p53, the JNK kinase pathway, or bcl-2 (29) in MG-132-induced apoptosis, although this in part contradicts an earlier report (30). Interestingly, clonogenic survival of HD-My-Z cells was completely abolished by maintaining them in the presence of concentrations of MG-132 as low as 0.5 μM. This suggests that the proteasome inhibition may be an effective new treatment modality for Hodgkin's disease, providing this level can be attained in humans without adverse toxicity.

There is surprisingly little knowledge on how Hodgkin cells respond to modalities that are classically used to treat

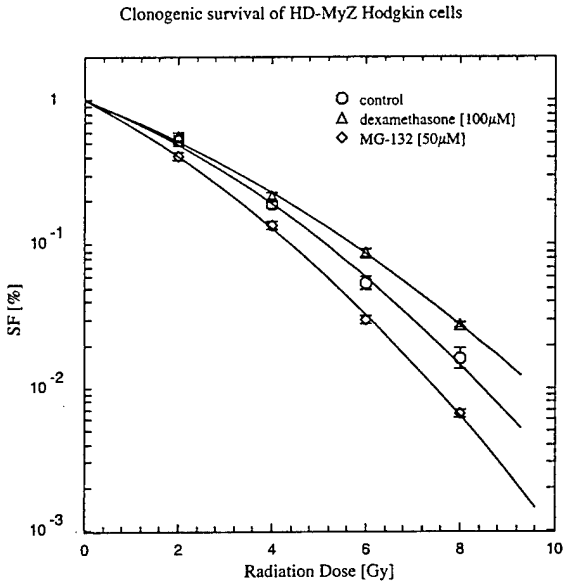


Fig. 8. Survival plots derived from colony forming assays with HD-My-Z cells after treatment with MG-132 or dexamethasone. Intrinsic radiosensitivity of HD-My-Z Hodgkin lymphoma cells was decreased ($p < 0.0001$, F-test) by inhibition of proteasome function by preincubation in MG-132 (50 μM) for 3 h before irradiation. Dexamethasone (100 μM) under the same conditions was a radioprotector ($p < 0.0001$, F-test) (control: $\alpha = 0.298 \pm 0.052$, β 7equal; 0.02893 $\pm$ 0.0072, $\alpha/\beta = 10.3$; MG-132: $\alpha = 0.3885 \pm 0.033$, $\beta = 0.03048 \pm 0.0041$, $\alpha/\beta = 12.7$; Dexamethasone: $\alpha = 0.2898 \pm 0.043$, $\beta = 0.01982 \pm 0.0048$, $\alpha/\beta = 14.6$).

this disease. Two such treatments are glucocorticoids and radiation. Glucocorticoids are a standard component of state-of-the-art chemotherapy protocols in Hodgkin's disease. Short-term treatment of HD-My-Z cells with glucocorticoids did not change their constitutive NF-κB activity. It also failed to induce apoptosis. If HD-My-Z cells are representative of the disease, these findings suggest that the tumor shrinkage seen after application of glucocorticoids in patients with Hodgkin's disease may be caused more by death of the inflammatory cells infiltrating the tumor than by death of tumor cells themselves. HD-My-Z was, however, isolated from the pleural effusion of a patient with relapsed and therapy-refractory Hodgkin's disease (19). Thus, this cell line might reassemble the phenotype of relapsing Hodgkin's disease rather than that of cases that are easily cured with standard therapy.

Radiation therapy often causes rapid shrinkage of bulky lymphomas after only a few treatment fractions. To our knowledge, there have been no reports on the radiosensitivity of Hodgkin cell lines. In this study, HD-My-Z cells were found to be moderately radiation resistant. In comparison, PC3 prostate cancer cells were 1.4 times more sensitive. Although it is difficult to extrapolate from these *in vitro* findings to the clinical situation, the results contrast with the doses of irradiation needed clinically to achieve tumor control in these two diseases. One possible explanation for the relative sensitivity of Hodgkin's lymphomas is that the number of malignant cells they contain is usually below 5%, and comparably lower doses would be required to achieve cure.

Interestingly, the proteasome inhibitor, MG-132, radiosensitized HD-My-Z cells. We had a similar result with SiHa cervical cancer cells (Pajonk *et al.*, unpublished data). Although MG-132 also inhibits calpain, there is strong evidence that the effects described in this study are the result of proteasome rather than calpain inhibition. First, MG-132 induces apoptosis in MOLT-4 and L5178Y cells, while Z-leu-leucinal, a specific calpain inhibitor, does not (31). Second, calpain is activated during apoptosis and calpain inhibition prevents apoptosis (32–34). Third, MG-132 is a relatively specific inhibitor for the proteasome (Ki for IκB 3 μM). Inhibition of calpain and cathepsin by peptide aldehydes occurs after doses much lower than those used in this study (5–12 nM) (35) and that are ineffective at inducing apoptosis. Finally, calpain is less important than the proteasome in eukaryotic proteolysis. Almost all short-lived (36) and 70–90% of all long-lived proteins (35, 37) are degraded by the 26s proteasome and its activity is up to 1,000-fold higher than the activity of calpain.

The mechanism for the radiosensitizing effect of proteasome inhibitors seen in this study also has yet to be elucidated. Proteasome inhibitors have been shown to activate caspase-3 indirectly (38). Caspase-3 targets DNA-PKcs, the catalytic subunit of DNA-dependent protein kinase responsible for repair of DNA double strand breaks (39), which is one possible pathway for radiosensitization. In contrast to the effect of proteasome inhibition, treatment with dexamethasone clearly protected HD-My-Z cells from irradiation, as has been reported for other cell lines (40, 41). There is no doubt that steroids are useful in combination with other chemotherapeutics in Hodgkin's disease because shrinkage of bulky tumors will enhance the effect of cytotoxic drugs by reperfusion and/or reoxygenation of malignant cells. However, if the cell line HD-My-Z can be taken as *pars pro toto* for Hodgkin's disease, steroids in combination with radiation therapy should be used with caution. In addition, the use of proteasome inhibitors, perhaps in combination with radiation therapy, has potential as a novel strategy for cases of Hodgkin's disease that resist conventional therapies.

REFERENCES

1. Aviles A, Delgado S. A prospective clinical trial comparing chemotherapy, radiotherapy and combined therapy in the treatment of early stage Hodgkin's disease with bulky disease. *Clin Lab Haematol* 1998;20:95–99.
2. Liao Z, Ha CS, Fuller LM, *et al.* Subdiaphragmatic stage I & II Hodgkin's disease: Long-term follow-up and prognostic factors. *Int J Radiat Oncol Biol Phys* 1998;41:1047–1056.
3. Gobbi PG, Pieresca C, Ghirardelli ML, *et al.* Long-term results from MOPPEBVCAD chemotherapy with optional

limited radiotherapy in advanced Hodgkin's disease. *Blood* 1998;91:2704–2712.
4. Specht L, Gray RG, Clarke MJ, *et al.* Influence of more extensive radiotherapy and adjuvant chemotherapy on long-term outcome of early-stage Hodgkin's disease: A meta-analysis of 23 randomized trials involving 3,888 patients. International Hodgkin's Disease Collaborative Group. *J Clin Oncol* 1998;16:830–843.
5. Glick JH, Young ML, Harrington D, *et al.* MOPP/ABV hybrid chemotherapy for advanced Hodgkin's disease significantly improves failure-free and overall survival: The 8-year results of the intergroup trial. *J Clin Oncol* 1998;16:19–26.
6. Josting A, Kàtay I, Rueffer U, *et al.* Favorable outcome of patients with relapsed or refractory Hodgkin's disease treated with high-dose chemotherapy and stem cell rescue at the time of maximal response to conventional salvage therapy (Dex-BEAM). *Ann Oncol* 1998;9:289–295.
7. Sweetenham JW, Taghipour G, Milligan D, *et al.* High-dose therapy and autologous stem cell rescue for patients with Hodgkin's disease in first relapse after chemotherapy: Results from the EBMT. Lymphoma Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant* 1997;20:745–752.
8. Bonfante V, Santoro A, Viviani S, *et al.* Outcome of patients with Hodgkin's disease failing after primary MOPP-ABVD [see comments]. *J Clin Oncol* 1997;15:528–534.
9. Marafioti T, Hummel M, Anagnostopoulos I, *et al.* Origin of nodular lymphocyte-predominant Hodgkin's disease from a clonal expansion of highly mutated germinal-center B cells [see comments]. *N Engl J Med* 1997;337:453–458.
10. Ohno T, Stribley JA, Wu G, *et al.* Clonality in nodular lymphocyte-predominant Hodgkin's disease [see comments]. *N Engl J Med* 1997;337:459–465.
11. Schwartz RS. Hodgkin's disease—time for a change [editorial; comment]. *N Engl J Med* 1997;337:495–496.
12. Bargou RC, Emmerich F, Krappmann D, *et al.* Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997;100:2961–2969.
13. Wang CY, Mayo MW, Korneluk RG, *et al.* NF-kappaB anti-apoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science* 1998;281: 1680–1683.
14. Wu MX, Ao Z, Prasad KV, *et al.* IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. *Science* 1998;281:998–1001.
15. Beg AA, Baltimore D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death [see comments]. *Science* 1996;274:782–784.
16. Baeuerle PA, Baltimore D. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell* 1988;53:211–217.
17. Baeuerle PA, Baltimore D. NF-kappa B: Ten years after. *Cell* 1996;87:13–20.
18. Wood KM, Roff M, Hay RT. Defective IκBα in Hodgkin cell lines with constitutively active NF-κB. *Oncogene* 1998;16: 2131–2139.
19. Bargou RC, Mapara MY, Zugck C, *et al.* Characterization of a novel Hodgkin cell line, HD-MyZ, with myelomonocytic features mimicking Hodgkin's disease in severe combined immunodeficient mice. *J Exp Med* 1993;177:1257–68.
20. Glas R, Bogyo M, McMaster JS, *et al.* A proteolytic system that compensates for loss of proteasome function. *Nature* 1998;392:618–622.
21. Iimuro Y, Nishiura T, Hellerbrand C, *et al.* NFkappaB prevents apoptosis and liver dysfunction during liver regeneration [published erratum appears in *J Clin Invest*1998;101:1541]. *J Clin Invest* 1998;101:802–811.
22. Bargou RC, Leng C, Krappmann D, *et al.* High-level nuclear NF-kappa B and Oct-2 is a common feature of cultured Hodgkin/Reed-Sternberg cells. *Blood* 1996;87:4340–4347.
23. Auphan N, DiDonato JA, Rosette C, *et al.* Immunosuppression by glucocorticoids: Inhibition of NF-kappa B activity through induction of I kappa B synthesis [see comments]. *Science* 1995;270:286–290.
24. Scheinman RI, Cogswell PC, Lofquist AK, *et al.* Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids [see comments]. *Science* 1995;270:283–286.
25. Pajonk F, Pajonk K, McBride WH. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells [In Process Citation]. *J Natl Cancer Inst* 1999;91:1956–1960.
26. Gruss HJ, Ulrich D, Braddy S, *et al.* Recombinant CD30 ligand and CD40 ligand share common biological activities on Hodgkin and Reed-Sternberg cells. *Eur J Immunol* 1995;25: 2083–2089.
27. Metkar SS, Naresh KN, Redkar AA, *et al.* CD40-ligation-mediated protection from apoptosis of a Fas-sensitive Hodgkin's-disease-derived cell line. *Cancer Immunol Immunother* 1998;47:104–112.
28. Gruss HJ, Hirschstein D, Wright B, *et al.* Expression and function of CD40 on Hodgkin and Reed-Sternberg cells and the possible relevance for Hodgkin's disease. *Blood* 1994;84: 2305–2314.
29. Herrmann JL, Briones F Jr, Brisbay S, *et al.* Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. *Oncogene* 1998;17:2889–2899.
30. Drexler HC. Activation of the cell death program by inhibition of proteasome function. *Proc Natl Acad Sci USA* 1997;94: 855–860.
31. Shinohara K, Tomioka M, Nakano H, *et al.* Apoptosis induction resulting from proteasome inhibition. *Biochem J* 1996; 317:385–388.
32. Waterhouse NJ, Finucane DM, Green DR, *et al.* Calpain activation is upstream of caspases in radiation-induced apoptosis. *Cell Death Differ* 1998;5:1051–1061.
33. Debiasi RL, Squier MK, Pike B, *et al.* Reovirus-induced apoptosis is preceded by increased cellular calpain activity and is blocked by calpain inhibitors. *J Virol* 1999;73:695–701.
34. Squier MK, Miller AC, Malkinson AM, *et al.* Calpain activation in apoptosis. *J Cell Physiol* 1994;159:229–237.
35. Rock KL, Gramm C, Rothstein L, *et al.* Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* 1994;78:761–771.
36. Ciechanover A. The ubiquitin-proteasome proteolytic pathway. *Cell* 1994;79:13–21.
37. Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. *J Biol Chem* 1996;271:27280–27284.
38. Wu LW, Reid S, Ritchie A, *et al.* The proteasome regulates caspase-dependent and caspase-independent protease cascades during apoptosis of MO7e hematopoietic progenitor cells. *Blood Cell Mol Dis* 1999;25:20–29.
39. Jeggo PA. Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat Res* 1998; 150:S80–S91.
40. Rutz HP, Mariotta M, von Knebel Doeberitz M, *et al.* Dexamethasone-induced radioresistance occurring independent of human papilloma virus gene expression in cervical carcinoma cells. *Strahlenther Onkol* 1998;174:71–74.
41. Mariotta M, Perewusnyk G, Koechli OR, *et al.* Dexamethasone-induced enhancement of resistance to ionizing radiation and chemotherapeutic agents in human tumor cells. *Strahlenther Onkol* 1999;175:392–396.

Anthracyclins, proteasome activity and inhibition of P-gp-mediated multi-drug-resistance

*[1¶]Frank Pajonk, [¶]Mirela Fekete, [¶]Judith Himmelsbach, [¶¶]Claudia Heilmann [¶¶¶]Melena Pervan and [¶¶¶]William H. McBride

[¶] Department of Radiotherapy, Radiological University Clinic, Freiburg, Germany

[¶¶] Department of Cardiovascular Surgery, University Clinic, Freiburg, Germany

[¶¶¶] Department of Radiation Oncology, Experimental Division, UCLA School of Medicine

Running title:

indexing phrases:

[1]Correspondence address: Department of Radiation Therapy, Molecular Radiation Biology Lab, Radiological University Clinic, Hugstetter Str. 55, 79106 Freiburg i. Brsg. Germany; Tel.: +49-761-270-3862, Fax: +49-761-270-3982; email: pajonk@uni-freiburg.de

*FP was supported by a grant of the Deutsche Forschungsgemeinschaft (DFG Pa 723/3-1)

Abstract

Background:

The multi-drug resistance gene 1 codes for the transmembrane efflux pump P-glycoprotein, responsible for an ATP-dependent export of structural unrelated compounds including many chemotherapeutic drugs. P-glycoprotein mediated multi-drug resistance is a common cause of chemotherapy failure. Drugs like cyclosporin A and verapamil can inhibit the efflux of chemotherapeutics, mediated by P-glycoprotein. However, since serum concentrations of these drugs, necessary to inhibit P-glycoprotein function, have severe side effects, they are still not part of standard chemotherapy regimens. Thus, there is an ongoing search for specific inhibitors of P-glycoprotein. Observations that cyclosporin A is also a proteasome inhibitor, led us to investigate a possible cross substrate specificity of P-glycoprotein and the 26s proteasome.

Material&Methods: Primary porcine heart fibroblasts were incubated with different doses of verapamil for 16 hours. VEGF mRNA expression was accessed by RT-PCR. Lysates from ECV 304 cells were incubated with different doses of verapamil, doxorubicin, daunorubicin, idarubicin, epirubicin, dactinomycin, topotecan, mitomycin C and gemcitabine. Chymotryptic 26s and 20s proteasome activity was measured using a fluorogenic peptide assay. Effects of doxorubicin on proteasome function in living cells was monitored in ECV304 cells, stable transfected with ubiquitin/green fluorescent protein fusion protein. Accumulation of daunorubicin in p-glycoprotein highly positive KB 8-5

cells was monitored in the presence of different doses of the proteasome inhibitor MG-132.

Results: Incubation of porcine heart fibroblast with verapamil caused a dose-dependent induction of VEGF mRNA expression. Incubation of crude cellular extracts of ECV 304 cells with Verapamil, doxorubicin, idarubicin, epirubicin, and dactinomycin led to a dose-dependent inhibition of 26s and 20s proteasome function. Incubation of crude cellular extracts of ECV 304 cells with daunorubicin inhibited 26s proteasome function in a dose-dependent manner but did not alter 20s proteasome activity. In contrast, incubation of crude cellular extracts of ECV 304 cells with topotecan, mitomycin C and gemcitabine left 26s and 20s proteasome activity unchanged. Incubation of KB 8-5 cells with different doses of MG-132 caused a dose-dependent accumulation of daunorubicin.

Conclusions: Our data indicates, that the 26s proteasome and P-glycoprotein have overlapping substrate specificities. Use of proteasome inhibitors in cancer therapy might not only increase radiosensitivity but could also sensitize tumors to standard chemotherapy protocols.

Introduction

Multi-drug-resistance (MDR) is a common reason for chemotherapy treatment failure in breast cancer, leukemia and non-Hodgkin lymphoma patients. Multi-drug-resistance is thereby often based on overexpression of the mdr1 gene. This gene codes for P-glycoprotein (P–gp) a 1280 amino acid transmembrane phosphoglycoprotein that functions as an ATP-dependent efflux pump. Numerous pre-clinical and clinical studies have been undertaken to overcome multi-drug-resistance and several substances have been identified, able to revert multi-drug-resistance *in-vitro* (reviewed in (1)). However, so far serum-concentrations of most MDR-modulating drugs required to revert multi-drug-resistance have unacceptable toxicity in-vivo. Therefore, combination protocols using cytotoxic drugs and P-gp inhibitors did not enter standard chemotherapy regimens. Insights into the mechanisms of interaction of these compounds with P-gp could be the basis for the development of more specific inhibitors.

Two of the most commonly used MDR-modulating substances are verapamil, cyclosporine A (CsA) and their derivates. It is remarkable that cyclosporine A has been recently identified as an inhibitor of the 26s proteasome (2). The 26s proteasome is a highly conserved multicatalytic protease responsible for ATP- and ubiquitin-dependent degradation of all short-lived and 70-90% of all long lived proteins including cyclin A, B and E, p21 and p27, p53, cJun, cFos, and IκB. As such a central protease the 26s proteasome controls the cell cycle, signal transduction pathways, apoptosis and major functions of the immune system.

The fact that CsA is a proteasome inhibitor gives rise to the assumption that most of the immunosuppressive properties of CsA are based on this inhibitory effect, causing a decrease of the diversity of MHC-I molecules on the cell surface of target cells (3) as well as apoptotic death of lymphocytes caused by inhibition of the transcription factor NF-κB (4).

Recently, N-benzyloxycarbonyl-L-leucyl-L-leucinal (zLLal), a calpain inhibitor, was reported to cause accumulation of ubiquitinated P-gp in K562 erythroleukemia cells while lactacystin, a potent a highly selective proteasome inhibitor had no effect, suggesting that calpain is involved in P-gp-mediated drug efflux in mdr1 positive cells. However, ubiquitination is usually restricted to 26s proteasome-mediated proteolysis. This led us to investigate a possible link between this protease and multi-drug-resistance.

Material and Methods

Cell culture

Primary porcine cardiac fibroblasts were obtained from fresh perivascular connective tissue from a porcine heart. The samples were minced and single pieces placed into 8.7cm^2 cell culture dishes. The flakes were squeezed under a glass cover slip, and supplemented with Dulbecco´s modified eagle medium (DMEM; Cell Concepts, Germany) with 10% fetal calf serum (FCS; Life Technologies), L-Glutamine 200mM (Cell Concepts), 200 IU/ml Penicillin/Streptomycin (Cell Concepts), 10 µg/ml Minocycline (ICN), and 10 µg/ml Amphotericin B (Bristol-Myers Squibb). Fibroblasts started to grow out of the tissue on the second day. Media was changed regularly 3 times a week. To increase the yield, some cover slips were taken off after 2 weeks and placed into new dishes. After reaching confluency, cells were trypsinized, transferred into cell culture flasks, and subsequently fed with DMEM, 10% FCS, 200 mM L-Glutamine, and 100 IU/ml Penicillin/Streptomycin. Cells up to the 12th passage were used for experiments. KB 8.5 human epitheloid carcinoma cells were a generous gift from Dr. Peter Hafkemeyer (University Clinic Freiburg, Germany). EVC 304 human bladder carcinoma cells were obtained from the German Microorganism And Tissue Culture Collection (DSMZ, Braunschweig). Cells were grown in 75 cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2. The medium used was DMEM medium (Sigma) supplemented with 10 % heat inactivated FCS (Sigma) and 1 % penicillin/streptomycin (Gibco BRL). Every 21 days P-gp-positive KB 8.5 cells were selected by addition of colchicin (10ng/ml,

Sigma). 24 hours before drug treatment cells were plated into 6-well plates (Costar) at a density of 10e6 cells/well.

Drug treatment

Stock solutions of all cytotoxic drugs were obtained from the hospital pharmacy, University Clinic Freiburg. MG-132 (Calbiochem) was dissolved in DMSO (10 mM), Lactacystin (Calbiochem) was dissolved in DMSO (1 mM) and small aliquots (10-30 µl) were stored at –20° C. At indicated times cells were washed twice with PBS and the growth medium was replaced by PBS containing Daunorubincin (16-2 µM), MG-132 (0.5-50 µM, 0.5% DMSO) or Lactacystin (5-2 µM) respectively. Control cells for MG-132 and Lactacystin treatment were subjected to DMSO treatment alone (0.5 %).

Proteasome function assays

Proteasome function was measured as described previously (20). Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1,000g for 5 minutes and 10,000g for 20 minutes. Protein concentration was determined by the BCA protocol (Pierce). 100µg protein of each sample was diluted with buffer I to a final volume of 1000µl. The fluorogenic proteasome substrate SucLLVY-7-amido-4-

methylcoumarin (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 80 µM in 1% DMSO. Cleavage activity was monitored continuously by detection of free 7-amido-4-methylcoumarin using a fluorescence plate reader (Gemini, Molecular Devices) at 380/460 nm and 37° C. Incubation of 7-amido-4-methylcoumarin (AMC, 2 µM) with cytotoxic drugs was carried out in buffer I for each sample in parallel and measurements of proteasome function were corrected when necessary.

Drug accumulation assay

Determination of total cellular daunorubicin content was carried out as described elsewhere with some minor modifications: Growth medium was replaced by PBS and cells were incubated at 37° C for 40 minutes. PBS was replaced by fresh PBS containing daunorubicin and MG-132 or daunorubicin alone. In some experiments cells were washed with PBS after daunorubicin treatment and incubated in PBS containing MG-132 for additional 40 minutes at 37° C. After drug treatment cells were washed twice with PBS, re-suspended in 400 µl 50/50 vol% ethanol (100%) / HCl (1M), vortexed and diluted with water to a final volume of 1.4 ml. Fluorescence was measured in quadruplicates of 200µl using a fluorescence plate reader (Gemini, Molecular Devices) at 480/575 nm.

Transfection

ECV 304 cells were maintained in DMEM (10 % FSC, 1% penicillin/streptomycin). 12 hours before transfection cells were trypsinized and

plated at a density of 250.000 cells/well into six-well plates. Cells were transfected with 5µg of a plasmid (pEGFP-N1, Clontech) coding for an ubiquitin-R-GFP fusion protein under control of a CMV promoter (5) (a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10% FSC, 1% penicillin/streptomycin) supplemented with 500µg/ml G418 (Sigma) and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-H, *FACSCalibur*, Becton Dickinson) using *CellQuest* Software before and after treatment with the proteasome inhibitor MG-132 (50µM, Calbiochem) for 10 hours at 37° C. Clone #10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

RT-PCR

First-strand cDNA was synthesized from 1 _g RNA by using Superscript II reverse transcriptase (Gibco BRL) and random hexamers (Pharmacia, Freiburg, Germany) as primers. Polymerase chain reaction (PCR) was carried out in an automatic thermal cycler (PTC 200, MJ Research). 1 _l (5%) of resulting cDNA was amplified in 25 _l PCR-reaction-mixtures using specific primers for porcine VEGF: VEGF forward: 5'-AGG AGA CCA GAA ACC CCA CG-3'; VEGF reverse: 5'-CTC AGT GGG CAC ACA CTC C-3'; ß-actin forward: 5'-GTC CCC ATC TAC GAG G-3'; ß-actin reverse: 5'-GCT CGT AGC TCT TCT CC-3'; (Genescan, Germany). PCR-conditions for VEGF: 94_ C – 53,4_ C - 72_ C, 18 cycles. PCR-

conditions for ß-actin: 18 cycles, 94_ C - 42_ C - 72_ C. All PCR-reactions were in linear range. The amplified products were analyzed on 0.7% agarose gels containing 1% ethidium bromide. Photographs were digitized using a flatbed scanner (CanoScan N650U, Canon). Densitometry was carried out using the NIH-Image Software for Macintosh Computers and the internal gel-macro2. Semi quantitative estimation was done by comparing VEGF to ß-actin mRNA expression.

Results

Verapamil is an inhibitor of 20s and 26s proteasome function

A report showing a pro-angiogenic effect of verapamil in a rat model (6) and the clinical observation from the second *Danish Verapamil Infarction Trial II* (DAVIT II) on 1775 patients, indicating that verapamil significantly reduced mortality and re-infarction rate (7), led us investigate a possible link between verapamil and the proteasome. In fact, cultures of primary porcine heart fibroblasts exhibited a dose-dependent 14fold increase of VEGF mRNA when treated with verapamil (0 to 200 μM) for 16 hours (Fig. 1). As gene expression of VEGF in general is dependent on stabilization of HIF-1α and in turn stabilization of HIF-1α usually occurs after inhibition of proteasome function, we decided to test if verapamil is a direct inhibitor of the 26s proteasome. Incubation of crude extracts of ECV304 cells with different concentrations of verapamil (0, 50, 100 and 200 μM) detected a dose-dependent inhibition of chymotryptic MG-132-sensitive 20s and 26s proteasome function, consistent with a direct inhibitory effect of verapamil on the proteasome (Fig. 2).

Anthracyclins inhibit 20s and 26s proteasome function in a dose-dependent manner

Vincristine, vinblastine and anthracyclins are classical substrates of P-gp (1). It has be recently shown, that vinblastine is a proteasome inhibitor (8). Since two of the most commonly used P-gp inhibitors cyclosporine A and verapamil (9) also

exhibited an inhibitory effect on 20s and 26s proteasome function we addressed the question if anthracyclins in general have an inhibitory effect on this protease. When crude extracts of ECV 304 cells were incubated with different doses of doxorubicin (Fig. 3A/B, 100 - 0 µM), daunorubicin (Fig. 3C/D, 100 - 0 µM), idarubicin (Fig. 3E/F, 100 - 0 µM), epirubicin (Fig. 3G/H, 100 - 0 µM) and dactinomycin (Fig. 3I/J, 10 – 0 µM) we observed a dose dependent inhibition of 26s proteasome function with IC_{50} values of 65.5 µM for doxorubicin, 13.7 µM for daunorubicin, 38.6 µM for idarubicin, 29.2 µM for epirubicin and 26 µM for dactinomycin. In contast, topotecan, mitomycin C and gemcitabine had no measurable effect on 26s proteasome function (data not shown). 20s proteasome function was inhibited by doxorubicin (IC_{50} 5.8 µM), idarubicin (IC_{50} 92 µM), epirubicin (IC_{50} 12.5 µM) and dactinomycin (IC_{50} 19.9 µM) but not by daunorubicin. In order to demonstrate the significance of this finding in living cells we incubated ECV304/10 cells, stable transfected with an expression plasmid for an ubiquitin-GFP fusion protein with doxorubicin (100 µM) for 12 hours. When analyzed by fluorescence microscopy the cells showed an accumulation of doxorubicin in a perinuclear structure while GFP accumulated in the whole cytoplasm in a dose-dependent manner, indicating an inhibition of proteasome function by doxorubicin in living cells (Fig. 3K).

MG-132 treatment reverts multi-drug-resistance in P-gp expressing KB 8-5 cells

The human epitheloid carcinoma cell line KB 8-5 is a well-characterized tumor cell line exhibiting multi-drug-resistance on the basis of P-gp expression. In our

initial experiments we observed that treatment of KB 8.5 cells with MG-132 (3.125 to 50μM) caused induction of apoptosis within 24 hours, indicating that MG-132 enters KB 8-5 cells and is not eliminated by P-gp function. This observation was in accordance with numerous studies reporting induction of apoptosis in cancer cells by proteasome inhibitors (10)(11-14). When KB 8-5 cells were treated with different doses of MG-132 and daunorubicin (10 μM) for 45 minutes we found a dose-dependent MG-132-induced cytoplasmic accumulation of daunorubicin (4-fold increase at 50μM MG-132, Fig. 4). However, treatment of KB 8-5 cells with the more specific proteasome inhibitor lactacystin failed to alter daunorubicin accumulation (data not shown). To exclude the possibility that lactacystin is a substrate for P-gp and is eliminated from cell by P-gp function we treated KB 8-5 cells with MG-132 or lactacystin for 3 hours and accessed the inhibitory effect of both drugs on chymotryptic 26s proteasome function by a fluorogenic peptide assay in crude cellular extracts. As expected, both inhibitors abolished 26s proteasome function (data not shown).

Discussion

P-gp mediated multi-drug-resistance is one of the most common causes of chemotherapy failure in cancer patients. Numerous *in-vitro* and *in-vivo* studies have been undertaken to circumvent MDR pharmacologically using P-gp modulating compounds like verapamil, cyclosporine A, reserpine, staurosporine, propafenone, phenoxazine, chloroquine, phenothiazine and their derivates (reviewed in (1)). However, non-P-gp-related site effects usually limit the clinical usage of these drugs in standard chemotherapy regimens. Insights into the mechanisms of MDR-modulation by these compounds could offer the basis for the development of specific P-gp inhibitors. Our observation that verapamil has inhibitory effects on the cleavage activity of the 26s proteasome and a recent report showing a comparable effect for vinbastine (8) led us investigate the effects of anthracyclins on the activity of this protease. We found a dose-dependent inhibitory effect on 26s proteasome function for all five anthracyclins tested. Additionally, except of daunorubicin, anthracyclins also inhibited 20s chymotryptic function in a dose-dependent manner.

A direct inhibitory effect of this class of chemotherapeutics on the proteasome is especially remarkable as tumor cells in general exhibit different expression pattern of proteasome subunits and a different distribution pattern of the proteasomes between cytoplasm and nucleus when compared with normal tissue cells (15-17). Further, anthracyclins are co-transported into the nucleus along with proteasomes (18, 19) and inhibition of proteasome function in tumor cells in general induces apoptosis (10) (11-14) and sensitizes the surviving cells to

ionizing radiation (20, 21). Direct inhibition of proteasome function might therefore be a major mechanism of this class of cytotoxic drugs.

Mechanistically, P-gp modulating drugs are either high-affinity substrates or inhibitors of ATP-dependent transport by P-gp (1). Our observation, that inhibitors of the 26s proteasome, the central protease of the major eukaryotic ATP-dependent protein degradation pathway, have inhibitory effects on P-gp function, could indicate that P-gp and the 26s proteasome have overlapping substrate specificities. Differences in proteasome subunit expression and patterns of proteasome distribution between malignant and normal cells can explain why specific proteasome inhibitors like PS-341 are clinically well tolerated (15-17). Thus, with proteasome inhibitors entering first clinical trials (22, 23), this class of substances might offer a new strategy to overcome P-gp-related MDR, combined with direct cytotoxic and radiosensitizing effects on tumors cells.

References

1. Ambudkar, S. V., Dey, S., Hrycyna, C. A., Ramachandra, M., Pastan, I., and Gottesman, M. M. Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol, *39:* 361-398, 1999.

2. Meyer, S., Kohler, N. G., and Joly, A. Cyclosporine A is an uncompetitive inhibitor of proteasome activity and prevents NF-kappaB activation. Febs Letters, *413:* 354-358, 1997.

3. Harding, C. V., France, J., Song, R., Farah, J. M., Chatterjee, S., Iqbal, M., and Siman, R. Novel dipeptide aldehydes are proteasome inhibitors and block the MHC-I antigen-processing pathway. J Immunol, *155:* 1767-1775, 1995.

4. Marienfeld, R., Neumann, M., Chuvpilo, S., Escher, C., Kneitz, B., Avots, A., Schimpl, A., and Serfling, E. Cyclosporin A interferes with the inducible degradation of NF-kappa B inhibitors, but not with the processing of p105/NF-kappa B1 in T cells. Eur J Immunol, *27:* 1601-1609, 1997.

5. Dantuma, N. P., Lindsten, K., Glas, R., Jellne, M., and Masucci, M. G. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat Biotechnol, *18:* 538-543., 2000.

6. Meirelles Pereira, L. M. and Mandarim-de-Lacerda, C. A. Effect of antihypertensive drugs on the myocardial microvessels in rats with nitric oxide blockade. Pathol Res Pract, *196:* 305-311, 2000.

7. Vaage-Nilsen, M., Hansen, J. F., Mellemgaard, K., Hagerup, L., Sigurd, B., and Steinmetz, E. Effect of verapamil on the prognosis of patients with early postinfarction electrical or mechanical complications. The Danish Verapamil Infarction Trial II (DAVIT II). Int J Cardiol, *48:* 255-258, 1995.

8. Piccinini, M., Tazartes, O., Mezzatesta, C., Ricotti, E., Bedino, S., Grosso, F., Dianzani, U., Tovo, P. A., Mostert, M., Musso, A., and Rinaudo, M. T. Proteasomes are a target of the anti-tumour drug vinblastine. Biochem J, *356:* 835-841, 2001.

9. Cardarelli, C. O., Aksentijevich, I., Pastan, I., and Gottesman, M. M. Differential effects of P-glycoprotein inhibitors on NIH3T3 cells transfected with wild-type (G185) or mutant (V185) multidrug transporters. Cancer Res, *55:* 1086-1091, 1995.

10. Shinohara, K., Tomioka, M., Nakano, H., Tone, S., Ito, H., and Kawashima, S. Apoptosis induction resulting from proteasome inhibition. Biochem J, *317:* 385-388, 1996.

11. Qiu, J. H., Asai, A., Chi, S., Saito, N., Hamada, H., and Kirino, T. Proteasome inhibitors induce cytochrome c-caspase-3-like protease-mediated apoptosis in cultured cortical neurons. J Neurosci, *20:* 259-265, 2000.

12. Pasquini, L. A., Besio Moreno, M., Adamo, A. M., Pasquini, J. M., and Soto, E. F. Lactacystin, a specific inhibitor of the proteasome, induces apoptosis and activates caspase-3 in cultured cerebellar granule cells. J Neurosci Res, *59:* 601-611, 2000.

13. Zhang, X. M., Lin, H., Chen, C., and Chen, B. D. Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochem J, *340:* 127-133, 1999.

14. Delic, J., Masdehors, P., Omura, S., Cosset, J. M., Dumont, J., Binet, J. L., and Magdelenat, H. The proteasome inhibitor lactacystin induces apoptosis and sensitizes chemo- and radioresistant human chronic lymphocytic leukaemia lymphocytes to TNF-alpha-initiated apoptosis [see comments]. Br J Cancer, *77:* 1103-1107, 1998.

15. Amsterdam, A., Pitzer, F., and Baumeister, W. Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cell cycle control. Proc Natl Acad Sci U S A, *90:* 99-103, 1993.

16. Kumatori, A., Tanaka, K., Inamura, N., Sone, S., Ogura, T., Matsumoto, T., Tachikawa, T., Shin, S., and Ichihara, A. Abnormally high expression of proteasomes in human leukemic cells. Proc Natl Acad Sci U S A, *87:* 7071-7075, 1990.

17. Kanayama, H., Tanaka, K., Aki, M., Kagawa, S., Miyaji, H., Satoh, M., Okada, F., Sato, S., Shimbara, N., and Ichihara, A. Changes in expressions of proteasome and ubiquitin genes in human renal cancer cells. Cancer Res, *51:* 6677-6685, 1991.

18. Kiyomiya, K., Matsuo, S., and Kurebe, M. Mechanism of specific nuclear transport of adriamycin: the mode of nuclear translocation of adriamycin-proteasome complex. Cancer Res, *61:* 2467-2471, 2001.

19. Kiyomiya, K., Matsuo, S., and Kurebe, M. Proteasome is a carrier to translocate doxorubicin from cytoplasm into nucleus. Life Sci, *62:* 1853-1860, 1998.

20. Pajonk, F., Pajonk, K., and McBride, W. Apoptosis and radisensitization of Hodgkin's cells by proteasome inhibition. Int.J.Radiat.Oncol, Biol, *47:* 1025-1032, 2000.

21. Russo, S. M., Tepper, J. E., Baldwin, A. S., Jr., Liu, R., Adams, J., Elliott, P., and Cusack, J. C., Jr. Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF-kappaB. Int J Radiat Oncol Biol Phys, *50:* 183-193., 2001.

22. Teicher, B. A., Ara, G., Herbst, R., Palombella, V. J., and Adams, J. The proteasome inhibitor PS-341 in cancer therapy. Clin Cancer Res, *5:* 2638-2645, 1999.

23. Adams, J., Palombella, V. J., and Elliott, P. J. Proteasome inhibition: a new strategy in cancer treatment. Invest New Drugs, *18:* 109-121., 2000.

Figures

Fig. 1

Verapamil induces expression of VEGF mRNA in porcine heart fibroblasts. Treatment of fibroblasts with 0, 50, 100 and 200µM verapamil for 16 hours caused a dose-dependent increase in VEGF mRNA expression (14fold at 200µM).

Fig. 2

Verapamil is an inhibitor of 26s proteasome function

Incubation of crude cellular extracts of ECV 304 cells with different doses of verapamil (50, 60, 80, 100, 200 µM) inhibited proteolysis of SucLLVY-AMC in a dose-dependent manner, indicating an inhibition of 26s proteasome function.

Fig. 3

Anthracyclins are inhibitors of proteasome function

(A-J) Incubation of crude cellular extracts of ECV 304 cells with different doses of doxorubicin (Fig. 3A/B, 100 - 0 µM), daunorubicin (Fig. 3C/D, 100 - 0 µM), idarubicin (Fig. 3E/F, 100 - 0 µM), epirubicin (Fig. 3G/H, 100 - 0 µM) and dactinomycin (Fig. 3I/J, 10 – 0 µM) caused an inhibition of 26s proteasome function. 20s proteasome was inhibited in case of doxorubicin, idarubicin, epirubicin, dactinomycin but not daunorubicin. (K) Incubation of

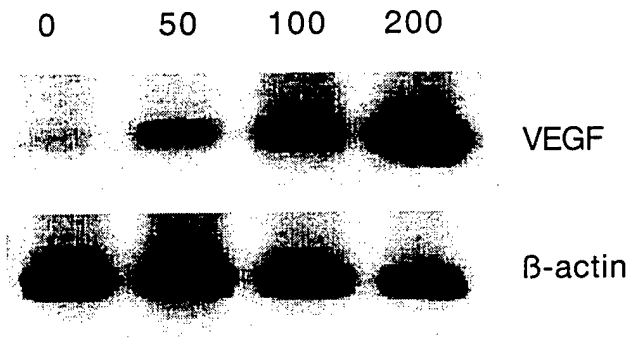
ECV 304 cells with doxorubicin (100μM, 16 h), stable transfected with an ubiquitin-GFP fusion protein, caused accumulation of GFP, indicating an inhibition of GFP in living cells.

Fig. 4

MG-132 treatment inhibits P-gp function

Incubation of P-gp expression KB 8-5 cells with increasing doses of MG-132 (50, 25, 12.5, 6.25, 0μM) caused a dose-dependent accumulation of daunorubicin, indicating inhibition of P-gp function by MG-132.

Fig. 1


0
50
100
200
VEGF
ß-actin

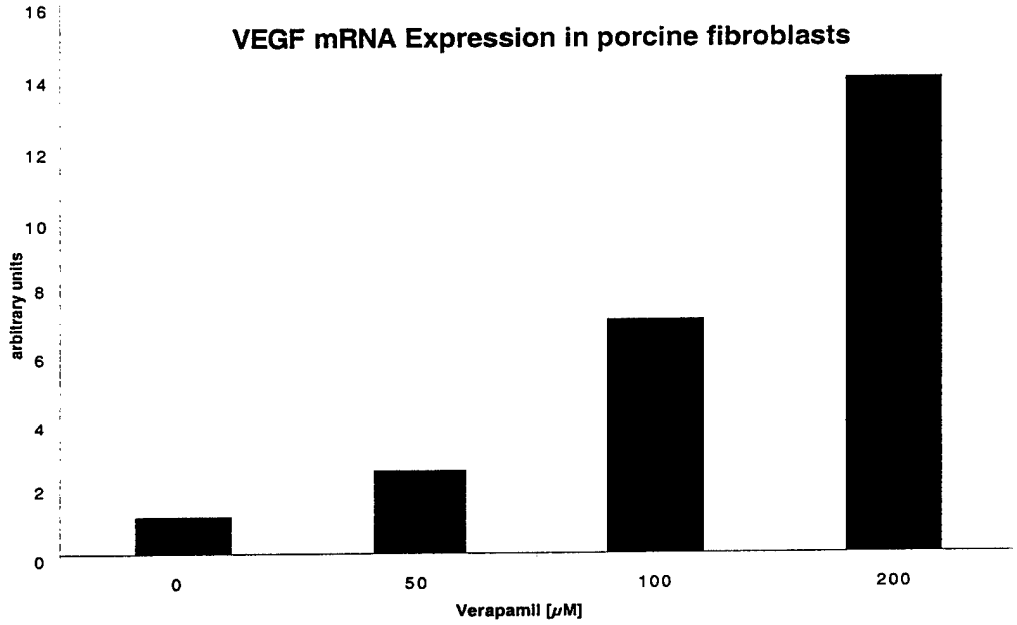

VEGF mRNA Expression in porcine fibroblasts
arbitrary units
16
14
12
10
8
6
4
2
0
0
50
100
200
Verapamil [µM]

Figure 2

Effect of Verapamil on 26 S Proteasome Function (ECV304 cells)

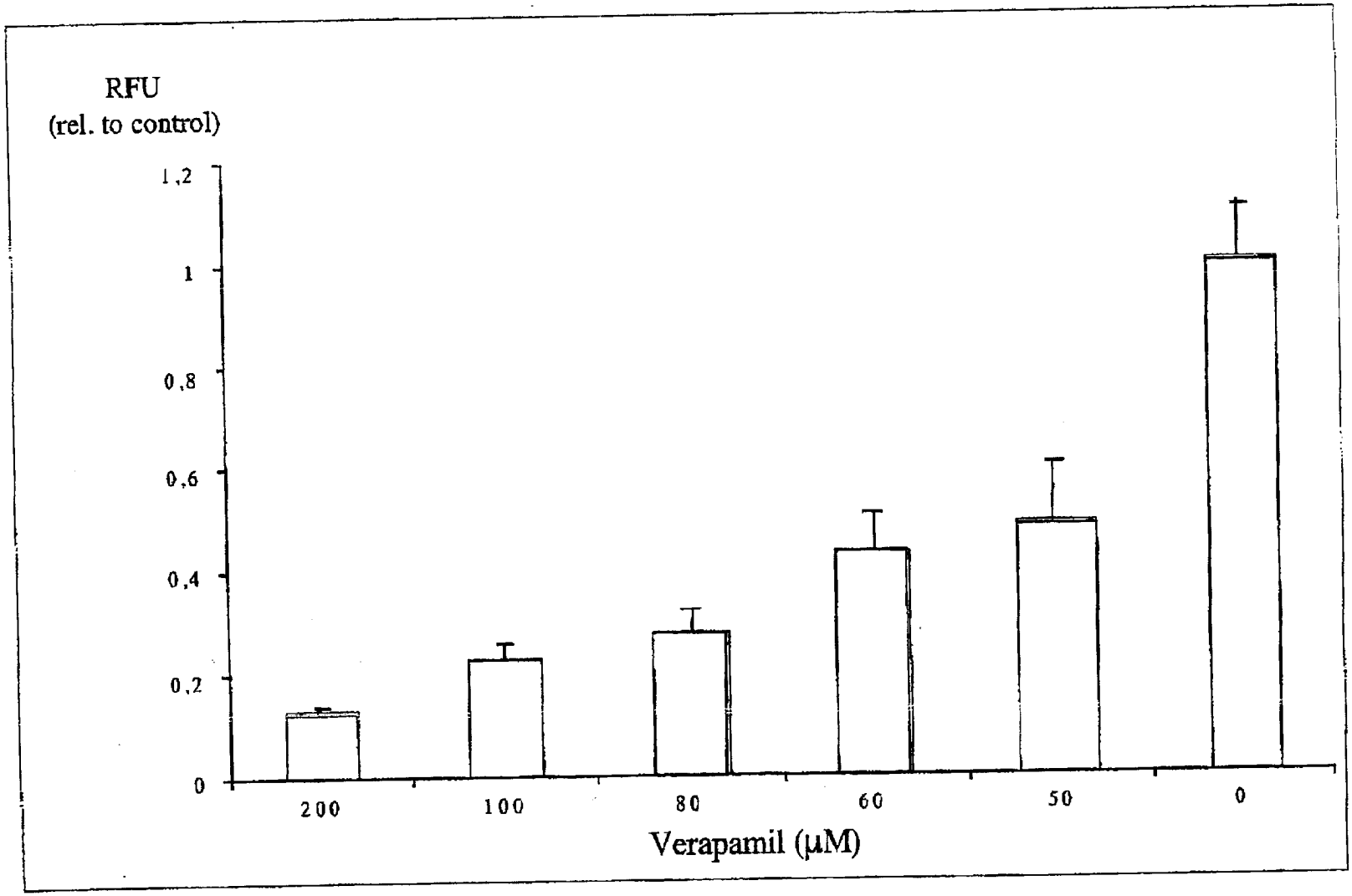
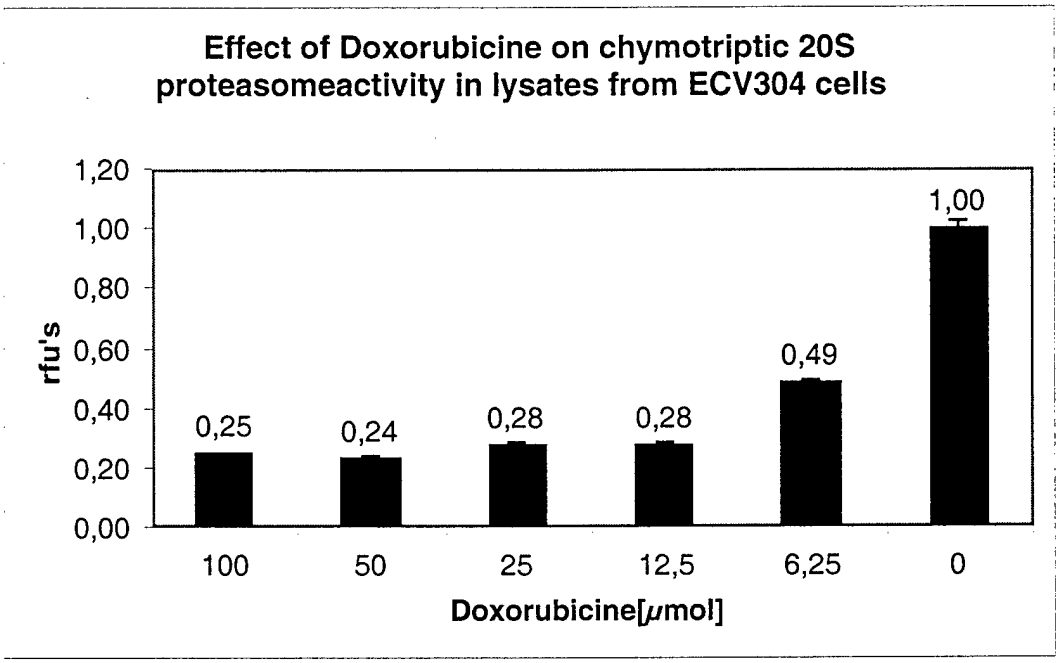


Fig 3

A


Effect of Doxorubicine on chymotriptic 20S proteasomeactivity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,25
0,24
0,28
0,28
0,49
1,00
100
50
25
12,5
6,25
0
Doxorubicine[μmol]

B

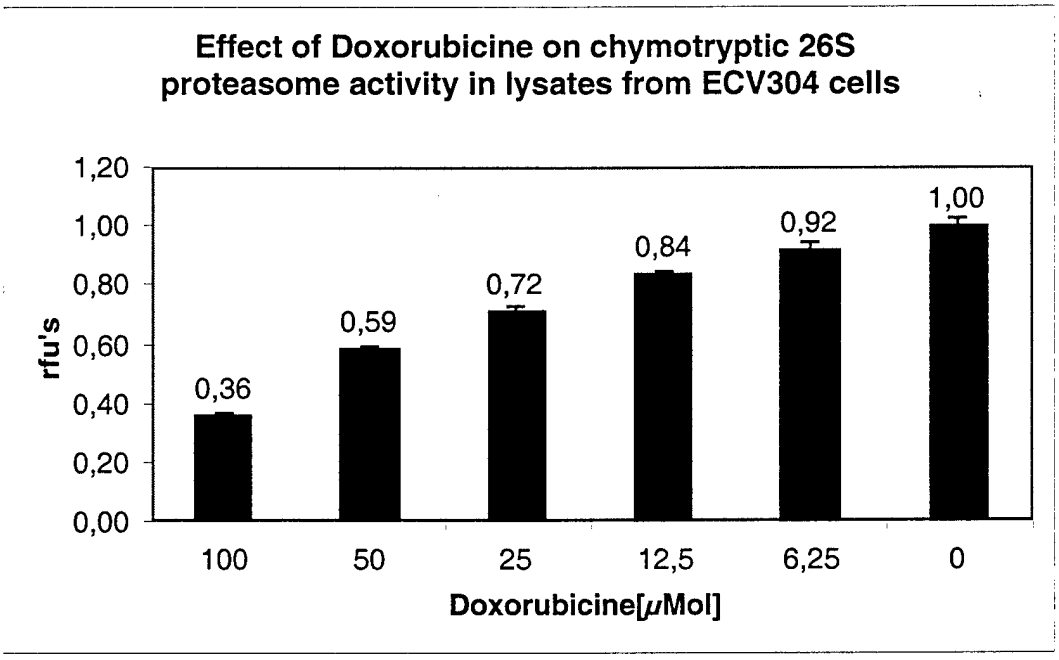
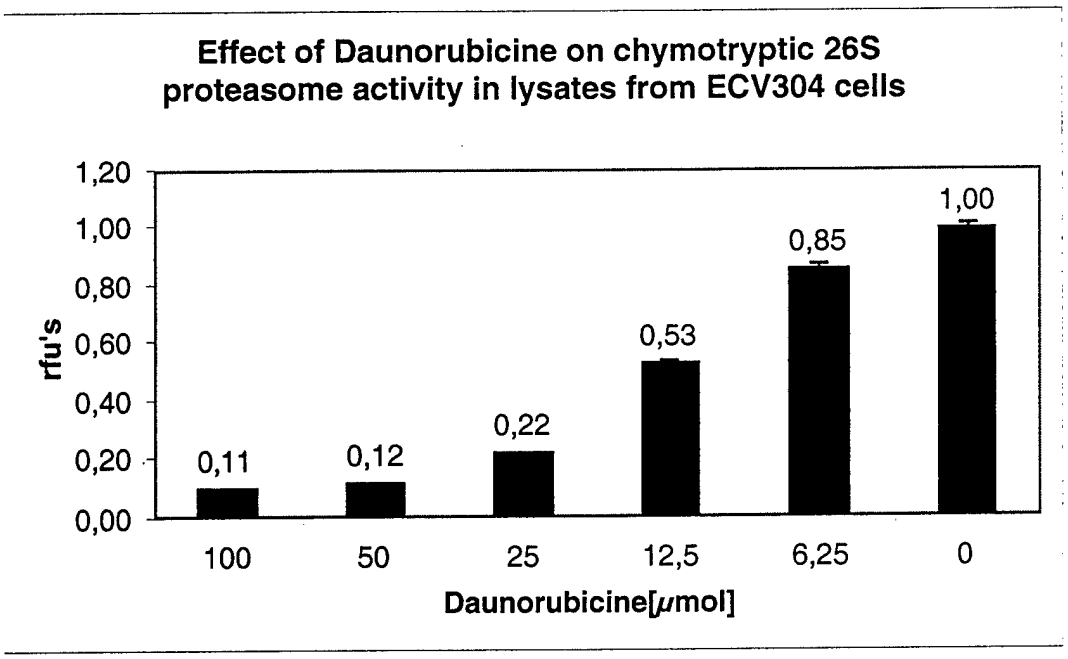

Effect of Doxorubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,36
0,59
0,72
0,84
0,92
1,00
100
50
25
12,5
6,25
0
Doxorubicine[μMol]

Fig. 3

C


Effect of Daunorubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,11
0,12
0,22
0,53
0,85
1,00
100
50
25
12,5
6,25
0
Daunorubicine[μmol]

D

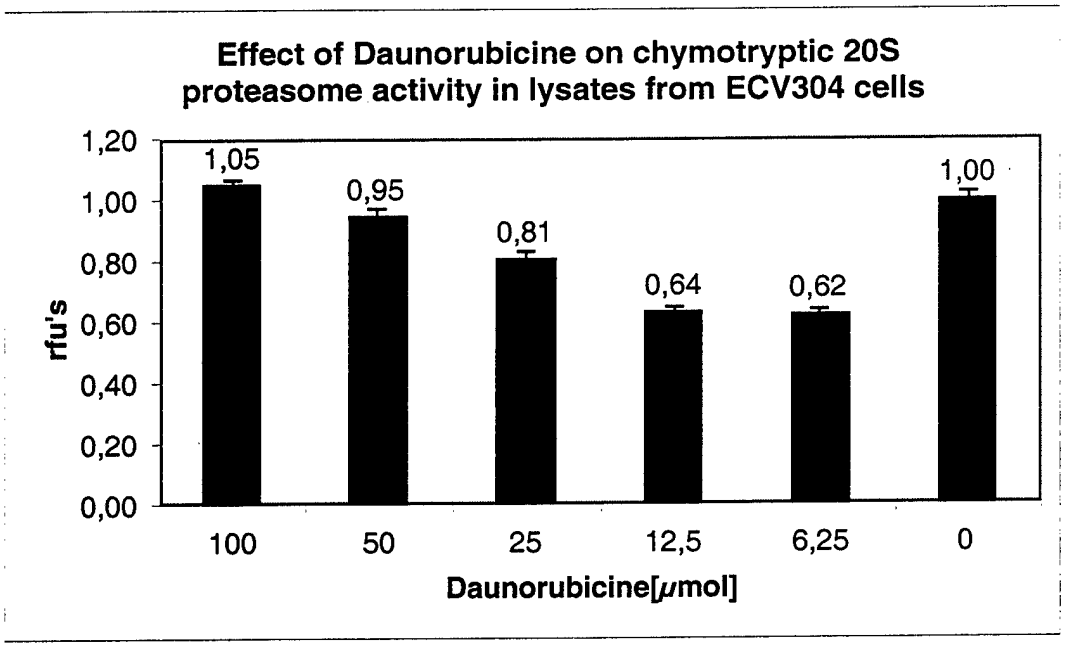
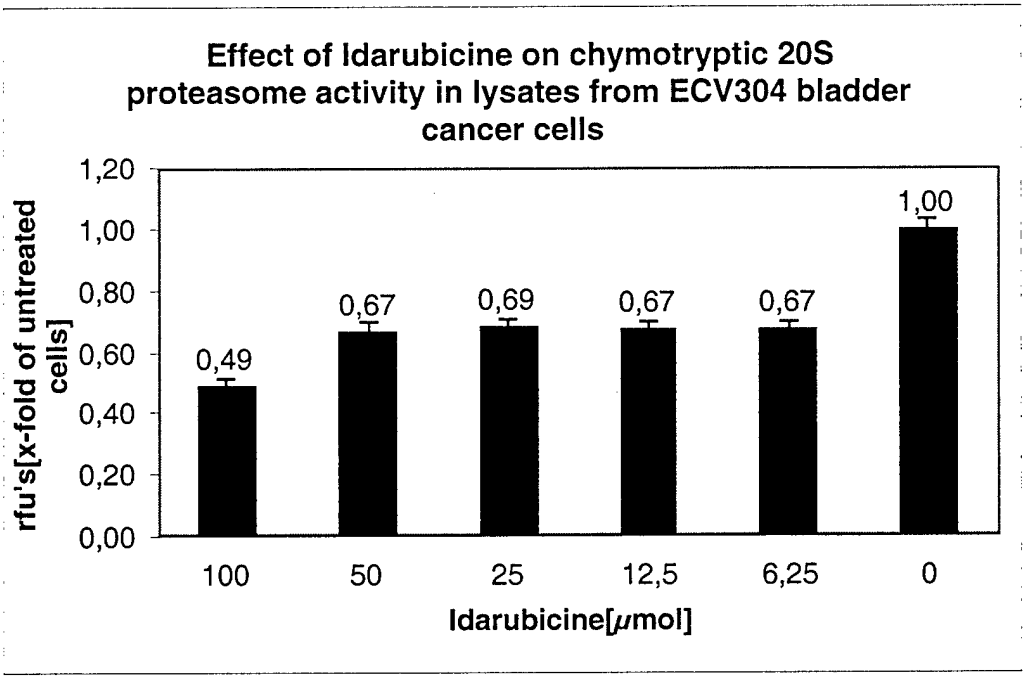

Effect of Daunorubicine on chymotryptic 20S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
1,05
0,95
0,81
0,64
0,62
1,00
100
50
25
12,5
6,25
0
Daunorubicine[μmol]

Fig. 3

E


Effect of Idarubicine on chymotryptic 20S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's[x-fold of untreated cells]
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,49
0,67
0,69
0,67
0,67
1,00
100
50
25
12,5
6,25
0
Idarubicine[µmol]

F

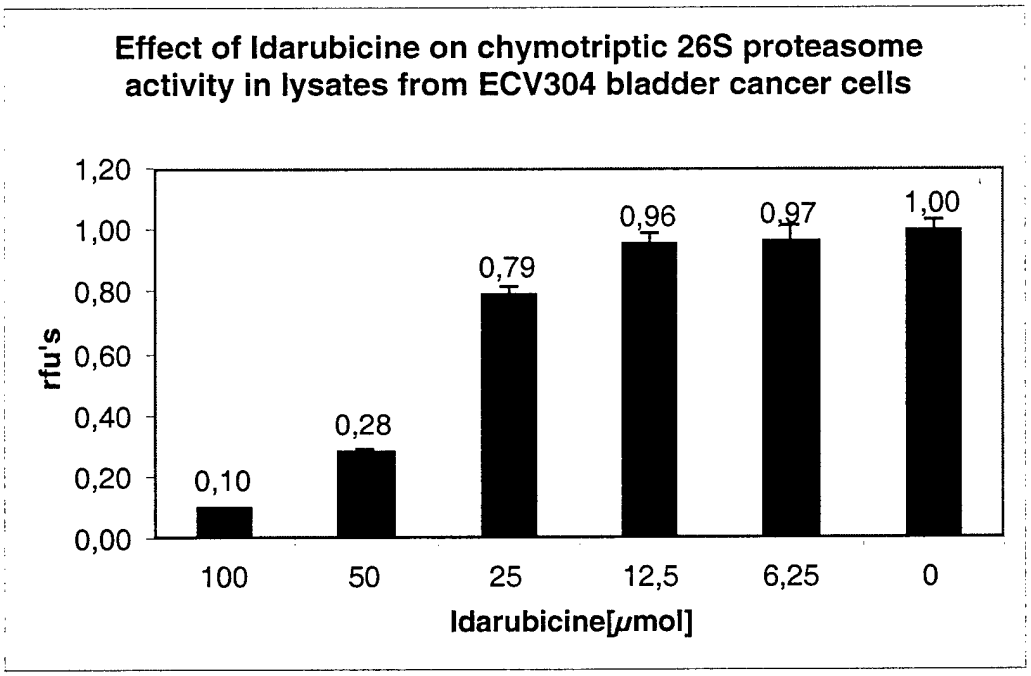
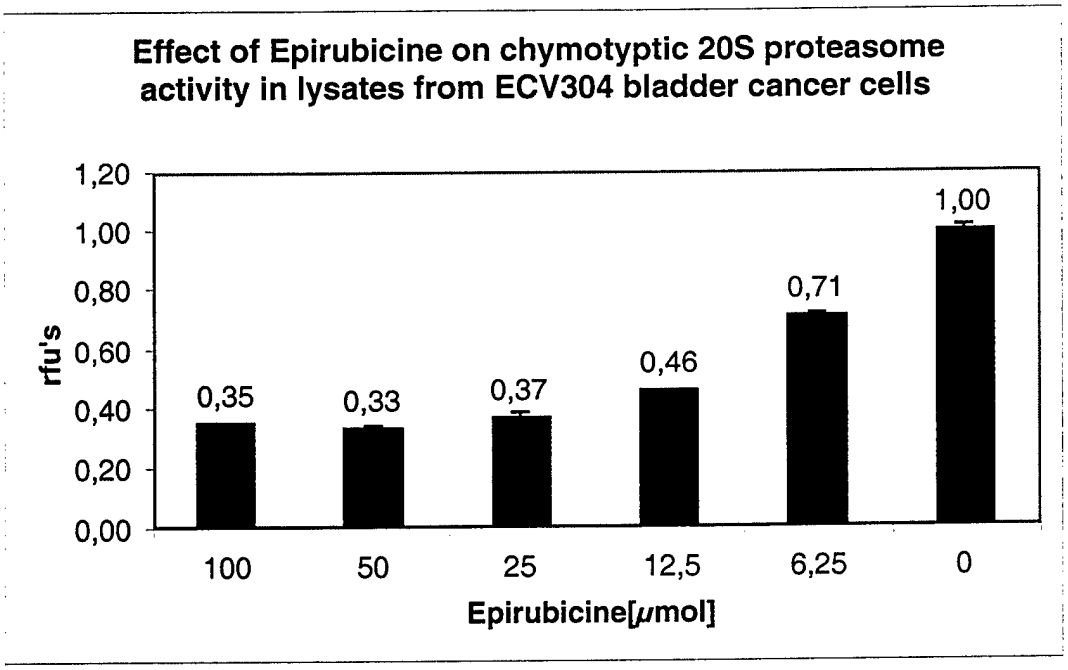

Effect of Idarubicine on chymotriptic 26S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,10
0,28
0,79
0,96
0,97
1,00
100
50
25
12,5
6,25
0
Idarubicine[µmol]

Fig. 3

G


Effect of Epirubicine on chymotyptic 20S proteasome activity in lysates from ECV304 bladder cancer cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,35
0,33
0,37
0,46
0,71
1,00
100
50
25
12,5
6,25
0
Epirubicine[μmol]

H

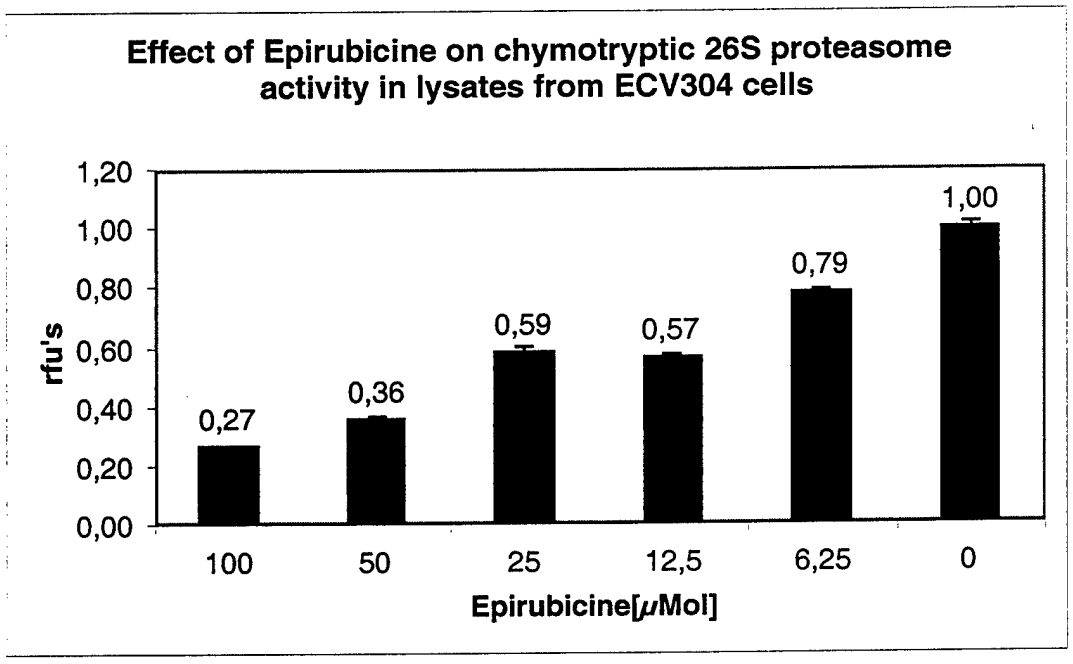
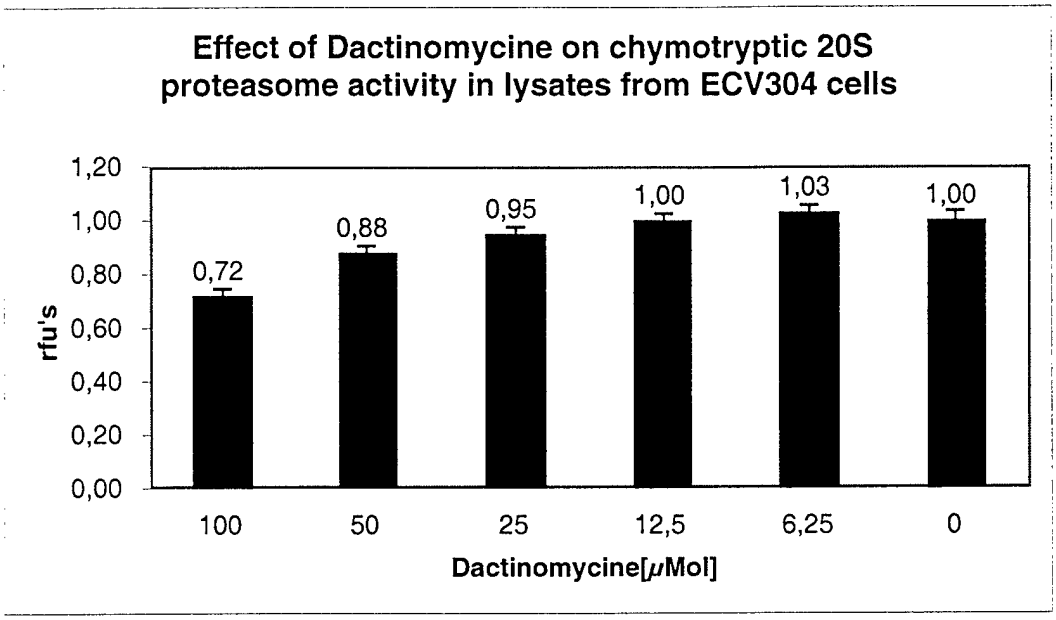

Effect of Epirubicine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,27
0,36
0,59
0,57
0,79
1,00
100
50
25
12,5
6,25
0
Epirubicine[μMol]

Fig. 3

I


Effect of Dactinomycine on chymotryptic 20S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,72
0,88
0,95
1,00
1,03
1,00
100
50
25
12,5
6,25
0
Dactinomycine[µMol]

J

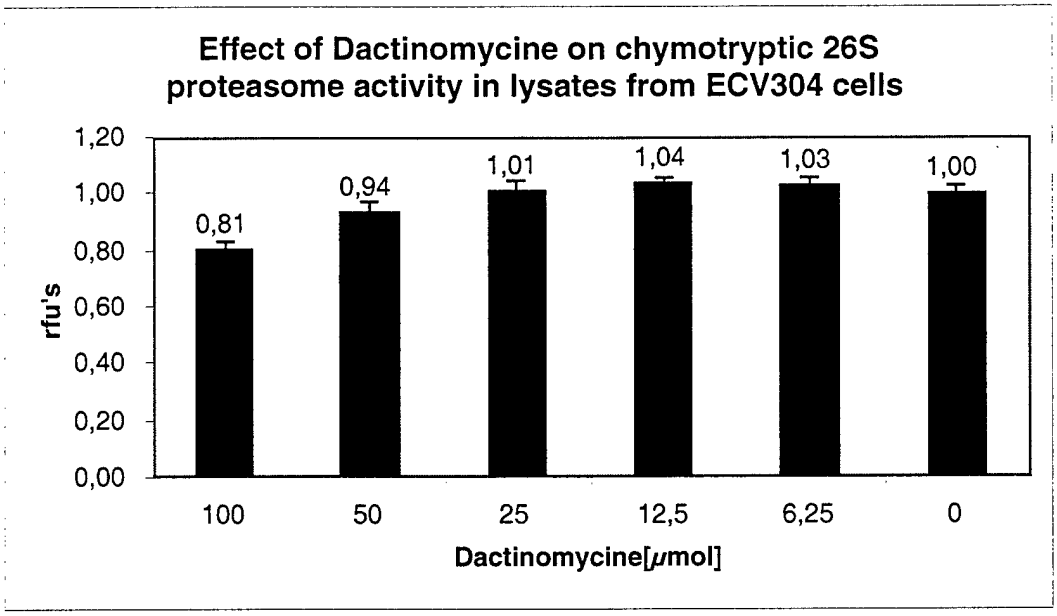
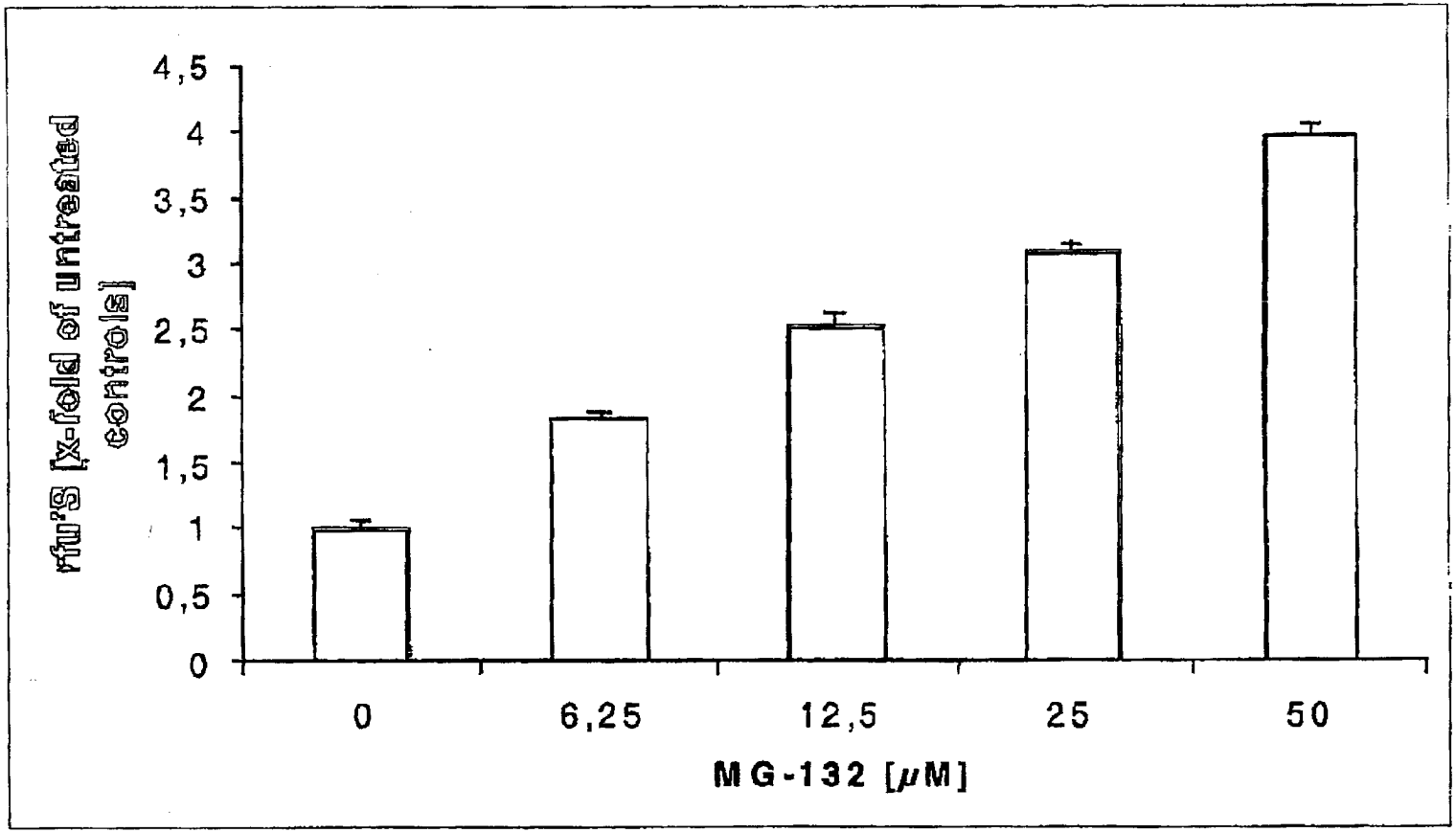

Effect of Dactinomycine on chymotryptic 26S proteasome activity in lysates from ECV304 cells
rfu's
1,20
1,00
0,80
0,60
0,40
0,20
0,00
0,81
0,94
1,01
1,04
1,03
1,00
100
50
25
12,5
6,25
0
Dactinomycine[µmol]

FIGURE 4

Accumulation of Daunorubicin in KB 8.5 Cells



KB 8.5 cells that overexpress P-gp cause dose dependent accumulation of drug after MG-132 treatment as measured by fluorescence

The HIV-1 protease inhibitor saquinavir inhibits proteasome function and induces apoptosis in human prostate cancer cells

***¶Frank Pajonk, ¶Judith Himmelsbach, ¶Katrin Riess, ¶Alfred Sommer and ¶¶William H. McBride**

¶ Department of Radiation Therapy, Radiological University Clinic Freiburg, Germany

¶¶ Department of Radiation Oncology, Experimental Division, UCLA School of Medicine

Keywords: HIV-I protease, proteasome, prostate cancer, ubiquitin, saquinavir

*Correspondence address: Department of Radiation Therapy, Radiological University Clinic, Hugstetter Str. 55, 79106 Freiburg i. Brsg. Germany; Tel.: +49-761-270-3862, Fax: +49-761-270-3982; email: pajonk@uni-freiburg.de

Abstract

Background: Cancer cells frequently show high constitutive activity of the anti-apoptotic transcription factor NF-κB, which results in their enhanced survival. Activation of NF-κB classically depends on degradation of its inhibitor IκBα by the 26s proteasome. Specific proteasome inhibitors induce apoptosis in cancer cells and, at non-lethal concentrations, sensitize cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs. Recently, the protease associated with the HIV-I virus has been found to have the same chymotrypsin-like cleavage sites as the proteasome. For this reason, we investigated whether the HIV-I protease inhibitor saquinavir can inhibit NF-κB activation, block 26s proteasome activity in prostate cancer cells and promote their apoptosis. Material&Methods: The effect of saquinavir on LPS/IFN-γ induced activation of NF-κB was assessed by gel-shift assays and by western analysis of corresponding IκBα-levels. Its effect on 20s and 26s proteasome activity was analyzed with a fluorogenic peptide assay using whole cell lysates from LnCaP, DU-145, and PC-3 prostate cancer cells pretreated with sequinavir for 9 hours. Proteasome inhibition in living cells was assessed using ECV 304 cells stably transfected with an expression plasmid for an ubiquitin/GFP fusion protein (ECV 304/10). Apoptosis was monitored morphologically and by flow cytometry.

Results: Saquinavir treatment prevented LPS/INF-γ induced activation of NF-κB in RAW cells and stabilized expression of IκBα. It inhibited 20s and 26s proteasome activity in lysates from LnCaP, DU-145 and PC-3 prostate cancer cells with an IC_{50} of 10 μM and caused accumulation of a ubiquitin/GFP transfusion protein in living ECV

304/10 cells. Incubation of human prostate cancer cells with saquinavir caused a dose-dependent induction of apoptosis.

Conclusions: We conclude that saquinavir, like ritonavir, inhibits proteasome activity in mammalian cells as well as acting on the HIV-I protease. As saquinavir induced apoptosis in human cancer cells, HIV-I protease inhibitors might become a new class of cytotoxic drugs, alone or in combination with radiation or chemotherapy.

Introduction

The human immunodeficiency virus type I (HIV-I) encodes for a protease required for the cleavage of the viral gag-pol polyprotein and its inhibition leads to release of noninfectious virus particles (*1*). The development of specific HIV-I protease inhibitors has revolutionized HIV therapy. At present, 5 different HIV-I protease inhibitors (PI's) –ritonavir, saquinivair, nelfinavir, indinavir and amprenavir– are clinically used (*2*). Bio-availability of at least ritonavir, saquinavir, and indinavir is limited by the fact that they are substrates, and in part inhibitors (*3*), of the same multi-drug resistance gene product (mdr-1) P-glycoprotein (*4,5*) that is a common cause for failure of chemotherapy in cancer patients.

The cleavage sites of action for HIV-I protease were once thought to be unique and distinct from those of mammalian proteases. However, recently, the 20s proteasome has been shown to cleave the same sites (*1*). This led us investigate whether saquinavir is an inhibitor of the 20s proteasome, as was previously reported for ritonavir (*6*). Proteasome inhibition might contribute to some of the effects of P.I.s that seem to be independent of virus inhibition, such as its immune-modulatory properties in HIV patients and its anti-tumoral action on HIV-associated Kaposi-sarcoma (*6*). We also examined whether saquinavir exhibits anti-tumoral effects in non-HIV-associated cancer of the prostate.

Material & Methods

Cell Culture

Cultures of PC3, LnCaP and DU-145 human prostate carcinoma cells (American Type Culture Collection, Maryland, USA), RAW 264.7 murine macrophages (a gift of Dr. G. Hildebrandt, Department of Radiation Oncology, University Clinic Leipzig), and ECV 304 human bladder carcinoma cells (DSMZ, Braunschweig) were grown in 75-cm^2 flasks (Greiner) at 37° C in a humidified atmosphere at 5 % CO_2/95 % air. Dulbecco's modified Eagle medium (DMEM, Cell Concepts, Freiburg) and RPMI 1640 medium (xxx) were supplemented with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (Gibco BRL) before use. Saquinavir (a generous gift of Dr. Christiane Moecklinghoff, Hoffmann La-Roche, Grenzach, Germany) was solubilized in ethanol/H_2O at a concentration 10mM and used at concentrations of 0, 20, 50, 60, 80 and 100μM. Controls received solvent only.

Transfection

ECV 304 cells were maintained in DMEM (10 %FSC, 1% penicillin/streptomycin). 12 hours before transfection cells were trypsinized and plated at a density of 250.000 cells/well into six-well plates. Cells were transfected with 5μg of a plasmid (pEGFP-N1, Clontech) coding for an ubiquitin-R-GFP fusion protein under control of a CMV promoter (7) (a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10%FSC, 1% penicillin/streptomycin) supplemented with 500μg/ml G418 (Sigma) and clones were

obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL2-A, *FACSCalibur*, Becton Dickinson) using *CellQuest* Software before and after treatment with the proteasome inhibitor MG-132 (50μM, Calbiochem) for 10 hours at 37°C. Clone #10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

Cell Extracts and Electrophoretic Mobility Shift Assays (EMSA)

Cellular extracts were prepared from normal and saquinavir-treated cells by dislodging the cells mechanically, washing with ice-cold phosphate-buffered saline (PBS), and lysing them in TOTEX buffer (20 mM HEPES, pH 7.9, 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 50μM phenylmethylsulfonyl fluoride (PMSF), and 90 trypsin inhibitor units (TIU's)/ml aprotinin) for 30 minutes on ice. The lysate was centrifuged at 12.000 x *g* for 5 minutes. Protein concentration in the supernatant was determined by the Micro BCA method (Pierce). Fifteen micrograms of protein were incubated for 25 minutes at room temperature with 2 μl of bovine serum albumin (10 μg/μl), 2 μl of dIdC (1 μg/μl), 4μl of Ficoll buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, and 0.1 mM PMSF), 2 μl of buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, and 0.1 mM PMSF), and 1 μl of the γ-^{32}P-ATP-labeled oligonucleotide (Promega, NF-κB: AGTTGAGGGGACTTTCCCAGG). A negative control was prepared for one sample by adding unlabeled oligonucleotide in 50-fold excess. Electrophoresis was carried out in native 4% polyacrylamide/0.5fold TBE

(Tris/boric acid/EDTA) gels. Dried gels were placed on a phosphor screen for 24 hours and analyzed on a phosphor imager (IPR 1500, Fuji).

Proteasome function assays

Proteasome function was measured as described previously (*8*) with some minor modifications. Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1000g for 5 minutes and the supernatent was further clarified by spinning at 10.000g for 20 minutes. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. 20 μg protein of each sample was diluted with buffer I to a final volume of 200 μl. To assess 26s function, fluorogenic proteasome substrate SucLLVY-MCA (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 80 μM in (0.8 % DMSO). To assess 20s function, buffer I was replaced by buffer containing SDS (20mM HEPES, pH 7.8; 0.5mM EDTA, 0.03% SDS)(REF). Proteolytic activity was monitored continuously using a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, 37° C) at 380/460 nm by release of the fluorescent group 7-amido-4-methylcoumarin (AMC).

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 % Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodium vanadate). Protein concentrations were determined using the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. 10 μg of protein were separated on SDS gel (0.1% SDS/12% polyacrylamide) and blotted to PVDF membranes at 4° C. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, 5% skim milk) for one hour at room temperature the membranes were incubated with a polyclonal antibody against IκBα (0.5μg/ml, BD Pharmingen) for one hour at room temperature. A secondary HRP-conjugated antibody and the ECLplus system (Amersham) were used for visualization.

Flow cytometry

For assessment of Ub-R-GFP expression, cells were trypsinized, pelleted (5 minutes, 500g) and washed twice in PBS. GFP-content was analyzed by flow cytometry using the *CellQuest* Software (FL1, *FACSCalibur*, Becton Dickinson). The DNA profiles of adherent and non-adherent cells in drug-treated populations were analyzed by flow cytometry using *CellQuest* Software (FL2-A, *FACSCalibur*, Becton Dickinson). Cells were washed in PBS and fixed overnight in ice-cold 75 % ethanol. The next day, cells were washed in PBS. The cell pellet was incubated with xmg/ml RNAse A and y% propidium iodide for 10 minutes and washed in PBS before examination.

Results

Saquinavir prevents activation of transcription factor NF-κB

NF-κB signaling is normally dependent on proteasomal degradation of the binding inhibitor IκBα and is prevented by treatment of cells with proteasome inhibitors (*9*). Murine RAW 264.7 macrophages respond strongly with NF-κB activation to treatment with LPS (0.1 μg/ml) and IFN-γ (100 U/ml) for 6 hours. This model was therefore used to determine if simultaneous addition of different concentrations of saquinavir (0, 6.25, 12.5, 25 and 50 μM) affected the activation process. When total cellular lysates were analyzed at 6 hours, we found a dose-dependent inhibitory effect of saquinavir on constitutive and LPS/IFN-γ induced increase of NF-κB DNA-binding activity (Fig. 1A) (it has a diagonal line on it – can you erase?). This inhibition coincided with accumulation of IκBα indicating involvement of the classical activation pathway of NF-κB by proteasome-dependent degradation of IκB (Fig. 1B). Similar results were observed for human PC3 prostate cancer cells (data not shown). IS THIS OK?

Saquinavir is an inhibitor of the 26s proteasome

We have previously shown that prostate cancer cells have been shown to express high constitutive levels of NF-κB that can be blocked by treatment with proteasome inhibitors (ref). The ability of saquinavir to inhibit proteasome function was examined by incubating cellular extracts of PC-3, DU-145 and LnCaP prostate cancer cells with different concentrations of the drug (100, 50, 25, 12.5, 6.25, 3,125, 1.6, 0 μM) for 45 minutes (TRUE?). Chymotryptic 20s and 26s proteasome activities were continuously monitored by the release of 7-amido-4-methylcoumarin (AMC) from the fluorogenic

proteasome substrate SucLLVY-AMC. Saquinavir inhibited the function of both 26s (Fig. 2 A, B, C) and 20s (Fig D) proteasome preparations in a dose dependent fashion. The IC_{50} was 10μM. In contrast, when PC-3 and LnCaP cells were treated with saquinavir supplemented media for 45 minutes (TRUE?) and the extracts then tested for activity, the IC_{50} was about 80μM (Fig 2 E and F). The difference in the ability of sequinavir to inhibit proteasome activity in whole cells and in extracts is probably due to its bioavailability, which may be adversely affected by saquinavir being a substrate of the mdr-1 gene product p-glycoprotein, which is expressed at high levels in prostate cancer cells (*10*)

To confirm that saquinavir can inhibit proteasome activity in cells, ECV 304 cells transfected with ubiquitin-R-GFP fusion protein (clone 10) were treated for 12 hours with media supplemented with different concentrations of saquinavir and incubated for additional 9 hours. Subsequent flow cytometric analysis revealed a dose dependent increase in GFP-positive cells from initially 0.38 % (0 μM) to 0.45 (20 μM), 14.8 (50 μM), 17.3 (60 μM), 20.9 (80 μM) and 19.7 (100 μM) (Fig. 3).

Saquinavir induces apoptosis in PC-3 prostate cancer cells

One of the many consequences of proteasome inhibition is induction of apoptosis (*11*). The ability of saquinavir treatment to achieve this endpoint was therefore tested. In PC-3 cells, 100μM induced apoptosis starting within 60 minutes. By 3 hours all cells showed typical morphological criteria of apoptosis (Fig. 4A). Most PC3 cells tolerated up to 50μM for over 24 hours, but 60μM induced considerable apoptosis by this time point. By 48 hours, PC3 cells showed an increase of the apoptotic (sub-G1) fraction from 10.4%

(0 μM) to 48.2% (50μM), 55.7% (60 μM) and 78.2% (80μM) (Fig. 4B). Saquinavir treatment also induced apoptosis in DU-145 cells (data not shown), although with delayed kinetics starting 24 hours after drug addition.

Discussion

The ubiquitin/26s proteasome pathway is the major non-lysosomal proteolytic pathway in mammalian cells. It is responsible for the degradation of all short-lived (*12*) and 70 to 90% of all long-lived proteins (*12,13*). Inhibition of proteasome function has been shown to induce apoptosis in cancer cells (*14-20*), and partial inhibition sensitizes surviving cells to the cytotoxic effects of ionizing radiation and chemotherapeutic drugs (*16,21*). Proteolysis by the ubiquitin/26s proteasome pathway is an important component of regulation of important cellular functions like signal transduction, cell cycle control, and immune responses (*22*). Interestingly, the 20s core unit of the proteasome is the only mammalian protease known so far to share specific cleavage action sites with the HIV-I protease, which may be a pathogenic mechanism adopted by the virus. A recent report indicated that the HIV-I protease inhibitor ritonavir inhibits 20s proteasome function (*23*). Here, we investigated the effect of the HIV-I protease inhibitor saquinavir, which is clinically less toxic (*1*), on 20s and 26s proteasome function and the possible physiological consequences of such inhibition in human cancer cells.

Prostate cancer cells in general show elevated constitutive DNA-binding activity of the anti-apoptotic transcription factor NF-κB (*24,25*) and we, and others, and we have demonstrated that inhibition of NF-κB induces apoptosis in cancer cells (*14,18,24,26*). NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the IκB family. Activation of this pathway is normally achieved by phosphorylation, polyubiquitination and subsequent degradation by the 26s proteasome of one of its most important inhibitors, IκBα. Degradation of IκBα frees NF-κB for translocation to the nucleus and

activation of its target genetic programs (reviewed in (*27*)). We have shown that the HIV-I protease inhibitor saquinavir blocks NF-κB activation in the murine RAW macrophage and human PC-3 prostate cancer cell lines and stabilizes IκBα in a dose-dependent fashion. Since activation of NF-κB is an important precondition for replication and persistence of HIV (*28*), this suggests a pathway for action of saquinavir that is independent of direct viral protease inhibition.

Saquinavir, like ritonavir, was shown to directly inhibit 20s and 26s proteasome function *in vitro*. Since inhibition of both 26S and 20S function showed similar drug concentration dependency, we conclude that it acts on the 20s core unit of the proteasome. Treatment of cells with sequinavir also inhibited proteasome function, although the IC_{50} was markedly higher, perhaps because saquinavir is a substrate for the multi-drug resistance (mdr-1) gene product P-glycoprotein, which is highly expressed in PC-3 human prostate cancer cells (ref). Physiological inhibition of proteasome function by saquinavir was demonstrated by the finding of a dose-dependent accumulation of the Ub-R-GFP reporter of proteasome function (*7*) in living ECV 304 cells stably transfected with this construct.

A physiological consequence of sequinavir treatment of PC-3 and DU-145 prostate cancer cells was apoptosis, which occurred with doses that were similar to those needed to inhibit proteasome function. These data are consistent with the hypothesis that saquinavir-induced apoptosis is the result of inhibition of proteasome function and the blocking of NF-κB activation. We have previously shown that inhibition of NF-κB activation in these cell lines by transduction with an IκB super-repressor gene also results in apoptosis (*16*).

The ubiquitin/26s proteasome has recently been identified as a novel target for cancer therapy (*16,19,29,30*). As inhibition of proteasome function in general sensitizes tumor cells to ionizing radiation (*16,21*) and HIV-I protease inhibitors have been shown to inhibit P-glycoprotein-mediated multi-drug resistance (*3*), HIV-I protease inhibitors might become new class of chemotherapeutic agents in radio-chemotherapy. The use of radiation therapy with 'baby-doses' of ritonavir might overcome the problem of low bio-availability of the drug (*31*).

Acknowledgements

We are grateful to Dr. M. Masucci, Karolinska Institute, Sweden, for providing the Ub-R-GFP plasmid and Dr. Ch. Moecklinghoff, Hoffmann La-Roche for providing saquinavir.

Figure legends

Fig. 1

Saquinavir inhibits activation of NF-κB

(A) RAW 264.7 macrophages were stimulated with LPS (0.1μg/ml) and IFN-γ (100U/ml) for 6 hours in the presence or absence of different concentrations of saquinavir and cell extracts were analyzed for NF-κB expression by EMSA. Lane 1: negative control with unlabeled oligonucleotide added in 50fold molar excess; lane 2: control unstimulated cells; lane 3: cells stimulated with LPS/IFN-γ in the absence of saquinavir; lane 4 to 7: cells stimulated with LPS/IFN-γ in the presence of saquinavir (lane 4: 50μM; lane 5: 25μM; lane 6: 12.5μM; lane 7: 6.25μM). **(B)** Western blot analysis for IκBα in cell extracts from RAW 264.7 macrophages stimulated with LPS (0.1μg/ml) and IFN-γ (100U/ml) for 6 hours in the presence or absence of different concentrations of saquinavir. Lane1…I am not sure of the treatments.

Fig. 2

Saquinavir is 20s proteasome inhibitor

Chymotryptic 26s and 20s proteasome activity in lysates from PC-3, DU-145 and LnCaP human prostate cancer cells. Cellular extracts from PC-3 (A), DU-145 (B) and LnCaP **(C)** cells were incubated with different doses of saquinavir. Chymotryptic 26s proteasome activity was assessed using a fluorogenic peptide assay with the specific proteasome substrate SucLLVY-MCA. Release of the fluorogenic compound 7-amido-4-methyl-cumarin was monitored continuously in a fluorescence plate reader (excitation 380 nm, emission 460 nm). The specificity of the cleavage reaction was demonstrated by

addition of the proteasome inhibitor MG-132 (Fig. 2 C, 50μM). Saquinavir inhibited chymotryptic 26s proteasome activity in all 3 cell lines in a dose-dependent manner. Saquinavir also inhibited chymotryptic 20s proteasome activity in LNCaP cells (Fig2 D), indicating an effect on the 20s core unit rather than on the 19s regulatory unit. The inhibitory effect was also observed when cells, rather than extracts, were incubated in growth media supplemented with different concentrations of saquinavir for 45 minutes (Fig. 2 E and F), indicating that saquinavir enters the cells, although the concentration of drug needed was higher.

Fig. 3

Saquinavir causes accumulation of the Ub-R-GFP reporter of proteasomal function

Flow cytometric analysis of ECV 304 cells stably transfected with Ub-R-GFP reporter of proteasomal function. Incubation with different concentrations of saquinavir for 9 hours causes a dose-dependent accumulation of Ub-R-GFP in cells. Positive cells are detected in the upper right quadrant. The number of positive cells and their average fluorescence are indicated.

Fig. 4

Saquinavir induces apoptosis in human prostate cancer cells

(A) DNA content of PC-3 prostate cancer cells incubated for 48 hours with different concentrations of saquinavir as determined by propidium iodide staining of ethanol-fixed cells. Incubation with saquinavir caused dose-dependent induction of apoptosis. The percentage of apoptotic cells in the pro-G1 peak is indicated. **(B)**

Incubation of PC3 cells with 100μM saquinavir caused a rapid induction of apoptosis within hours. Cell showed membrane blebbing and chromatin condensation as early as 3 hours after start of incubation.

Literature

(*1*) Flexner C. HIV-protease inhibitors. N Engl J Med 1998;338:1281-92.

(*2*) Eron JJ, Jr. HIV-1 protease inhibitors. Clin Infect Dis 2000;30 Suppl 2:S160-70.

(*3*) Drewe J, Gutmann H, Fricker G, Torok M, Beglinger C, Huwyler J. HIV protease inhibitor ritonavir: a more potent inhibitor of P- glycoprotein than the cyclosporine analog SDZ PSC 833. Biochem Pharmacol 1999;57:1147-52.

(*4*) Lee CG, Gottesman MM, Cardarelli CO, Ramachandra M, Jeang KT, Ambudkar SV, et al. HIV-1 protease inhibitors are substrates for the MDR1 multidrug transporter. Biochemistry 1998;37:3594-601.

(*5*) Srinivas RV, Middlemas D, Flynn P, Fridland A. Human immunodeficiency virus protease inhibitors serve as substrates for multidrug transporter proteins MDR1 and MRP1 but retain antiviral efficacy in cell lines expressing these transporters. Antimicrob Agents Chemother 1998;42:3157-62.

(*6*) Lebbe C, Blum L, Pellet C, Blanchard G, Verola O, Morel P, et al. Clinical and biological impact of antiretroviral therapy with protease inhibitors on HIV-related Kaposi's sarcoma. Aids 1998;12:F45-9.

(*7*) Dantuma NP, Lindsten K, Glas R, Jellne M, Masucci MG. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat Biotechnol 2000;18:538-43.

(*8*) Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL. A proteolytic system that compensates for loss of proteasome function. Nature 1998;392:618-22.

(*9*) Li N, Karin M. Ionizing radiation and short wavelength UV activate NF-kappaB through two distinct mechanisms. Proceedings of the National Academy of Sciences of the United States of America 1998;95:13012-7.

(*10*) Theyer G, Schirmbock M, Thalhammer T, Sherwood ER, Baumgartner G, Hamilton G. Role of the MDR-1-encoded multiple drug resistance phenotype in prostate cancer cell lines. J Urol 1993;150:1544-7.

(*11*) Shinohara K, Tomioka M, Nakano H, Tone S, Ito H, Kawashima S. Apoptosis induction resulting from proteasome inhibition. Biochem J 1996;317:385-8.

(*12*) Ciechanover A. The ubiquitin-proteasome proteolytic pathway. Cell 1994;79:13-21.

(*13*) Lee DH, Goldberg AL. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem 1996;271:27280-4.

(*14*) Herrmann JL, Briones F, Jr., Brisbay S, Logothetis CJ, McDonnell TJ. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. Oncogene 1998;17:2889-99.

(*15*) Zhang XM, Lin H, Chen C, Chen BD. Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochem J 1999;340:127-33.

(*16*) Pajonk F, Pajonk K, McBride W. Apoptosis and radisensitization of Hodgkin's cells by proteasome inhibition. Int.J.Radiat.Oncol, Biol 2000;47:1025-32.

(*17*) Kitagawa H, Tani E, Ikemoto H, Ozaki I, Nakano A, Omura S. Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. FEBS Lett 1999;443:181-6.

(*18*) McDade TP, Perugini RA, Vittimberga FJ, Jr., Callery MP. Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. Surgery 1999;126:371-7.

(*19*) Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999;59:2615-22.

(*20*) An B, Goldfarb RH, Siman R, Dou QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 1998;5:1062-75.

(*21*) Russo SM, Tepper JE, Baldwin AS, Jr., Liu R, Adams J, Elliott P, et al. Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF-kappaB. Int J Radiat Oncol Biol Phys 2001;50:183-93.

(*22*) Rolfe M, Chiu MI, Pagano M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. Journal of Molecular Medicine 1997;75:5-17.

(*23*) Schmidtke G, Holzhutter HG, Bogyo M, Kairies N, Groll M, de Giuli R, et al. How an inhibitor of the HIV-I protease modulates proteasome activity. J Biol Chem 1999;274:35734-40.

(*24*) Pajonk F, Pajonk K, McBride WH. Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. J Natl Cancer Inst 1999;91:1956-60.

(*25*) Palayoor ST, Youmell MY, Calderwood SK, Coleman CN, Price BD. Constitutive activation of IkappaB kinase alpha and NF-kappaB in prostate cancer cells is inhibited by ibuprofen. Oncogene 1999;18:7389-94.

(*26*) Bargou RC, Emmerich F, Krappmann D, Bommert K, Mapara MY, Arnold W, et al. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. Journal of Clinical Investigation 1997;100:2961-9.

(*27*) Baeuerle PA, Baltimore D. NF-kappa B: ten years after. Cell 1996;87:13-20.

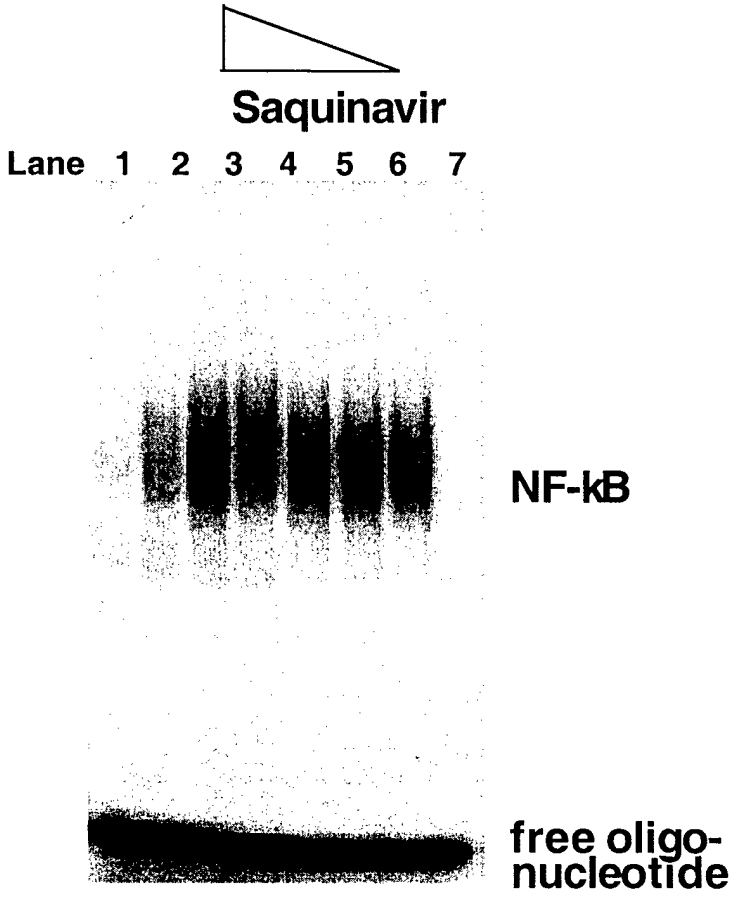
(*28*) Jacque JM, Fernandez B, Arenzana-Seisdedos F, Thomas D, Baleux F, Virelizier JL, et al. Permanent occupancy of the human immunodeficiency virus type 1 enhancer by NF-kappa B is needed for persistent viral replication in monocytes. J Virol 1996;70:2930-8.

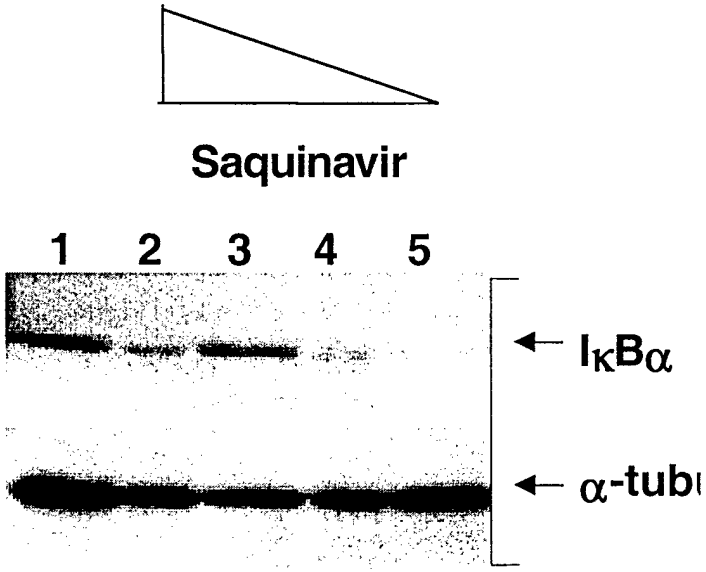
(*29*) Adams J, Palombella VJ, Elliott PJ. Proteasome inhibition: a new strategy in cancer treatment. Invest New Drugs 2000;18:109-21.

(*30*) Teicher BA, Ara G, Herbst R, Palombella VJ, Adams J. The proteasome inhibitor PS-341 in cancer therapy. Clin Cancer Res 1999;5:2638-45.

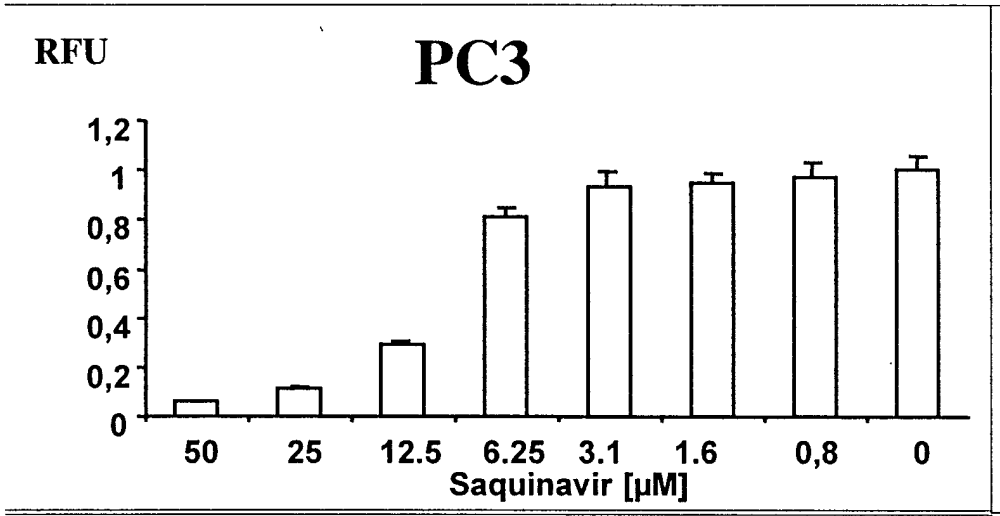
(*31*) Kurowski M, Muller M, Donath F, Mrozikiewicz M, Mocklinghoff C. Single daily doses of saquinavir achieve HIV-inhibitory concentrations when combined with baby-dose ritonavir. Eur J Med Res 1999;4:101-4.

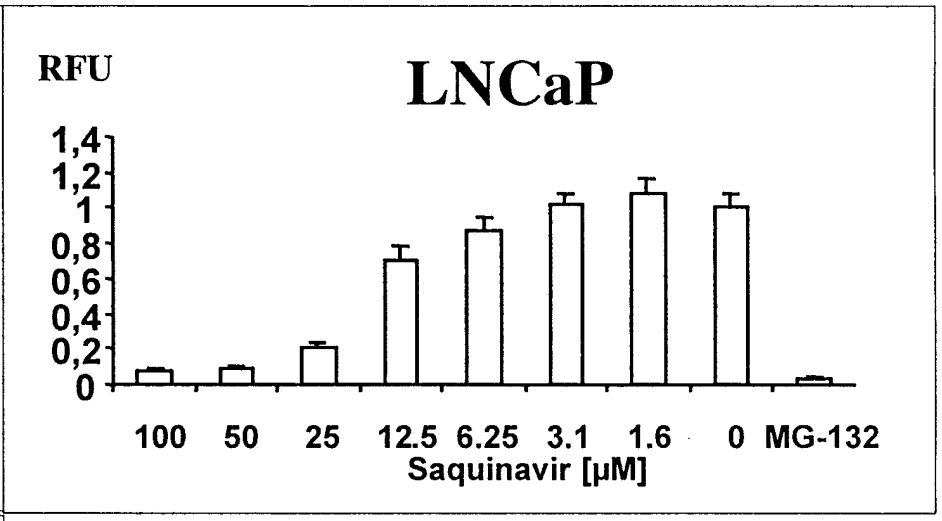
Effect of Saquinavir on NF-kB Activity

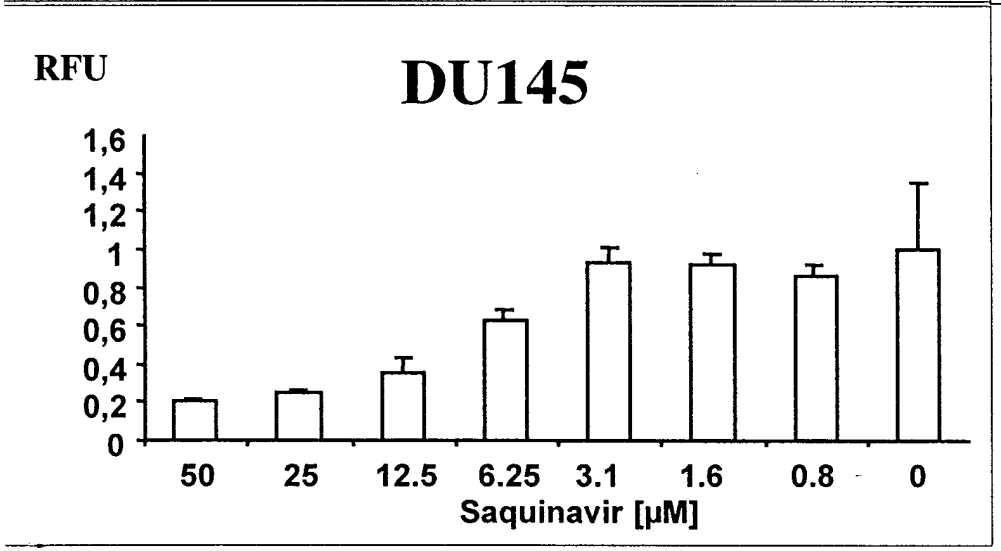




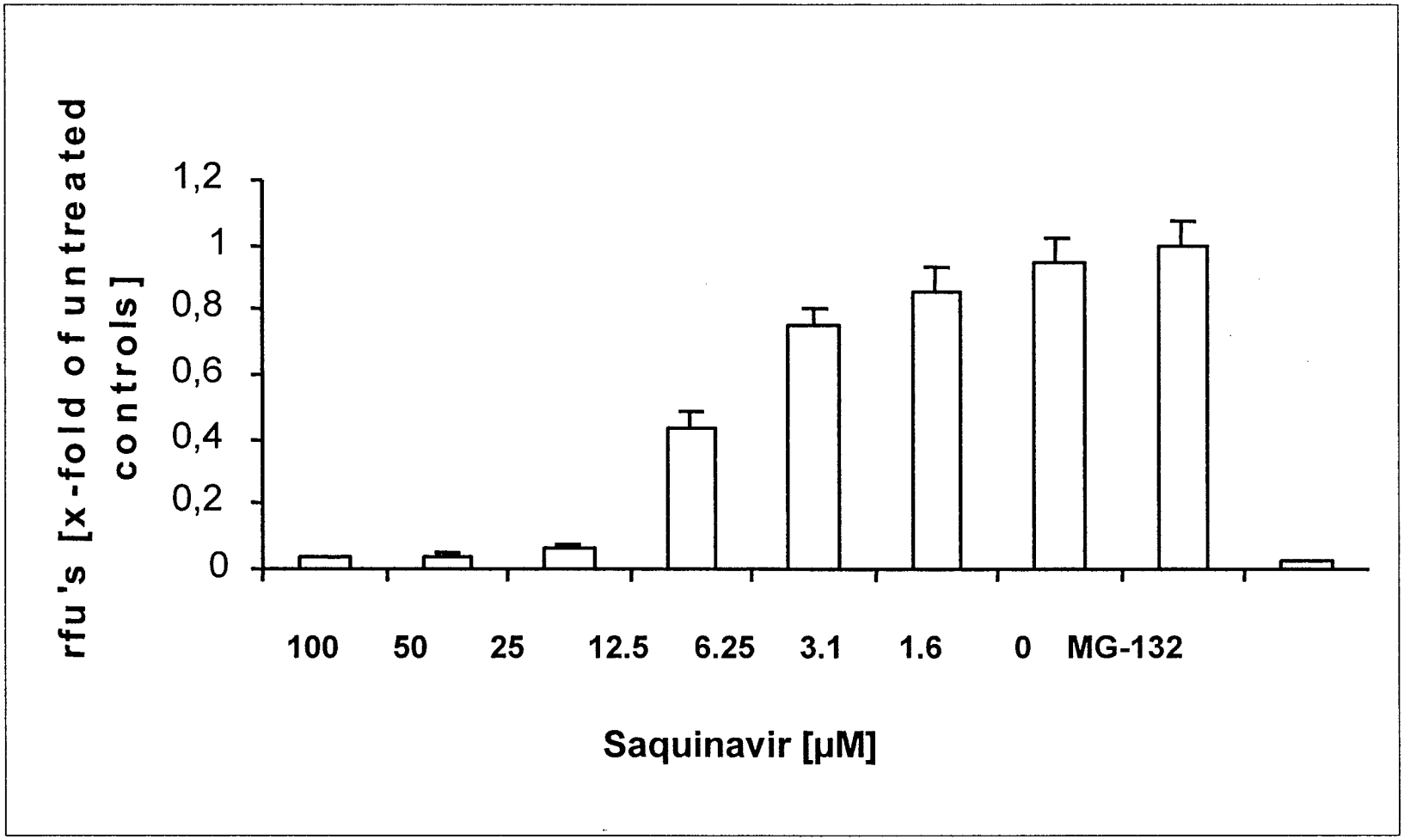
Effect of Saquinavir on 26S Proteasome Activity



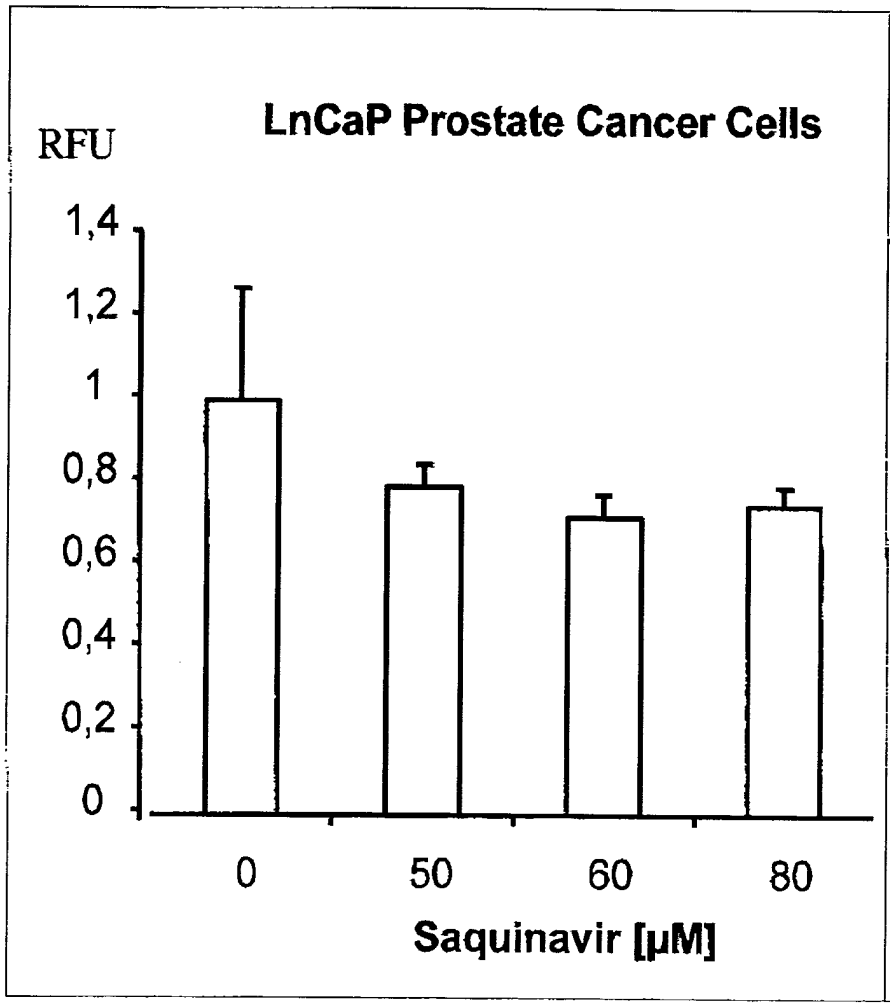


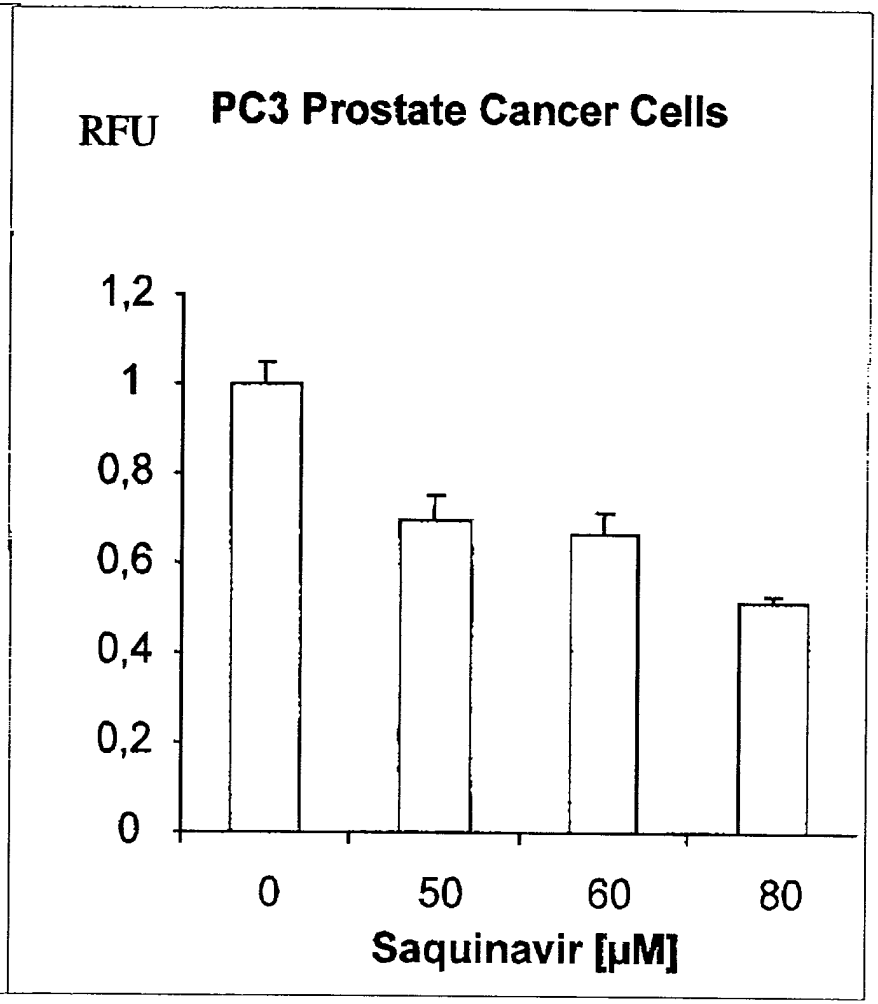


Effect of Saquinavir on 20s Proteasome Activity in LNCaP Cell Extracts

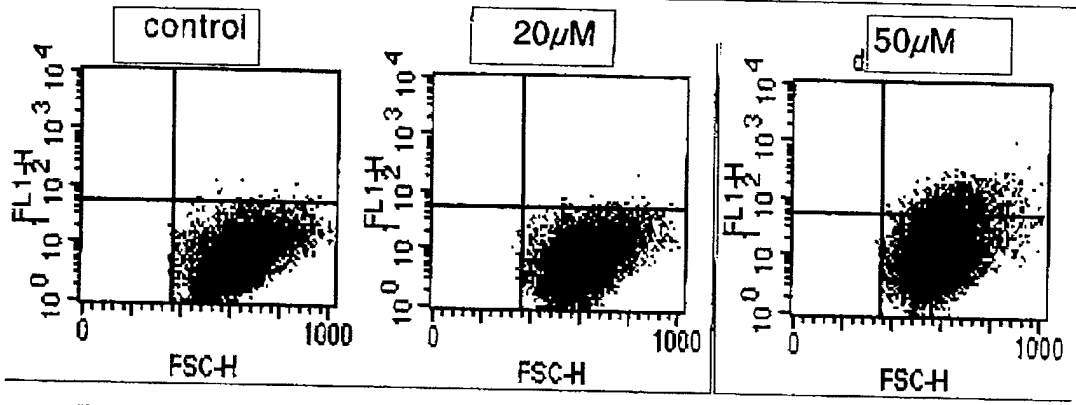


Effect of Saquinavir on 26s Proteasome function





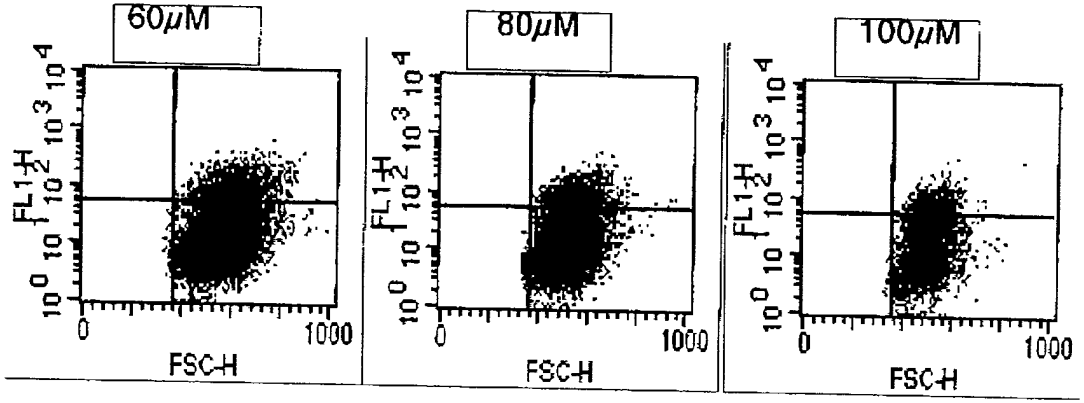
Sequinavir Causes Accumulation of Ub-GFP in ECV304 Cells



Positive: 0,38 %
Mean FL: 8,2

Positive: 0,45 %
Mean FL: 8,8

Positive: 14,8 %
Mean FL: 27,4



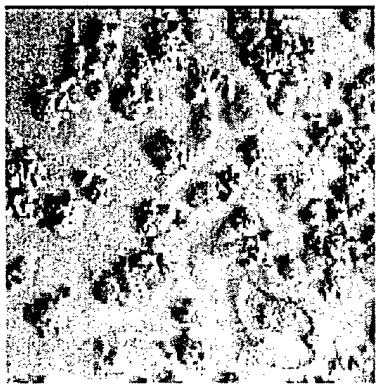
Positive: 17,3 %
Mean FL: 36,6

Positive: 20,9 %
Mean FL: 33,3

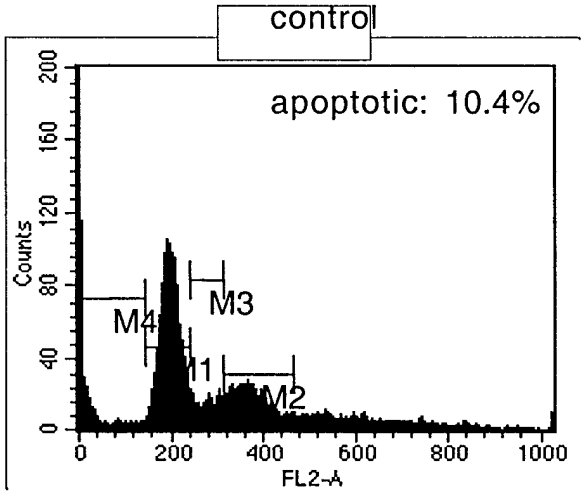
Positive: 19,7 %
Mean FL: 31,3

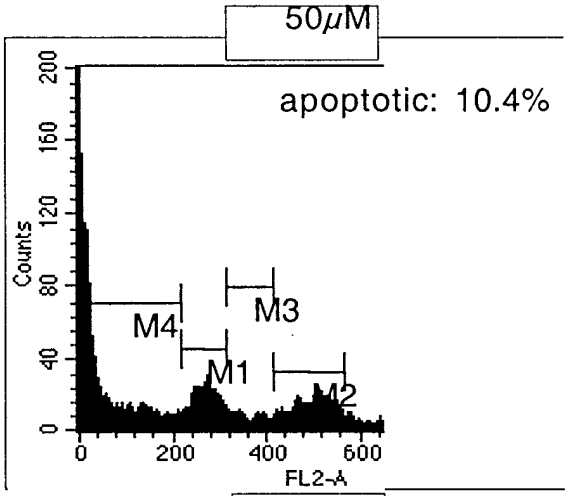
Sequinavir Induces Apoptosis in PC3 Cells

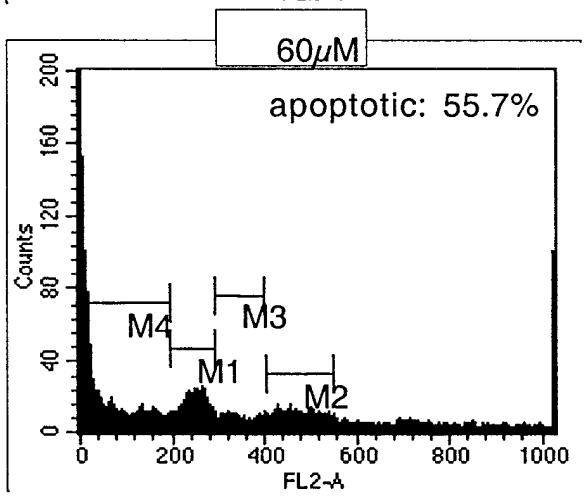
control

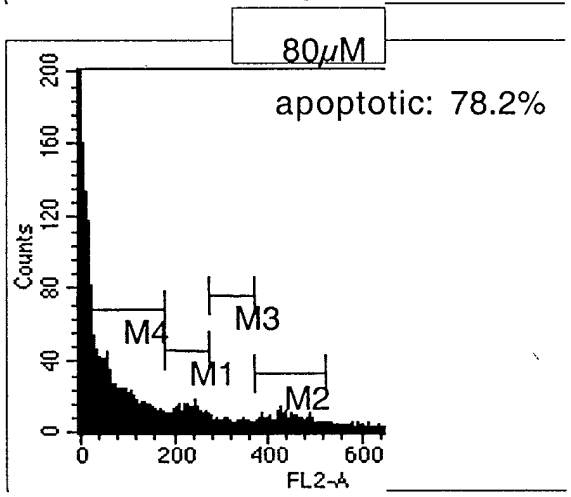


Saquinavir 100μM/3h









N-acetyl-L-cysteine inhibits 26s proteasome function – implications for effects on NF-κB activation

§†Frank Pajonk, †Katrin Riess, †Alfred Sommer and *William H. McBride

† Department of Radiation Therapy, Radiological University Clinic Freiburg, Germany

*Department of Radiation Oncology, Experimental Division, UCLA School of Medicine

Running Title: NAC is a 26s proteasome inhibitor

§Correspondence address: Department of Radiation Therapy, Radiological University Clinic, Hugstetter Str. 55, 79106 Freiburg i. Brsg. Germany; Tel.: +49-761-270-3862, Fax: +49-761-270-3982; email: pajonk@uni-freiburg.de

Abstract

Ionizing radiation shares with cytokines, such as TNF-α, an ability to generate free radicals in cells and activate downstream pro-inflammatory responses through NF-κB-dependent signal transduction pathways. Support for the role of free radicals in triggering such responses comes from the use of free radical scavengers like N-acetyl-L-cysteine (NAC). The nature of the link between free radical generation and NF-κB activation is however unclear. In this study, we explore the possibility that scavenging of free radicals by NAC might not be the mechanism by which it inhibits NF-κB activation, but rather that NAC acts through inhibition of proteasome function.

The effect of NAC on the chymotryptic function of the 26s and 20s proteasome complex was measured in extracts from EVC304 bladder carcinoma cells by assessing degradation of fluorogenic substrates. NAC inhibited 26s but not 20s proteasome activity, suggesting that it interferes with 19s regulatory subunit function. NAC blocked radiation-induced NF-κB activity in ECV 304 cells and RAW 264.7 macrophages, as measured by a gel shift assay, at doses that inhibited proteasome activity. This provides a possible mechanism whereby NAC could block NF-κB activation and affect the expression of other molecules that are dependent on the ubiquitin/proteasome system for their degradation, other than by scavenging free radicals.

Keywords: NF-κB, radiation effects, N-acetyl-L-cysteine, radical scavenger

Introduction

The free radical scavenger N-acetyl-L-cysteine (NAC) is used clinically for a broad spectrum of indications including mucolysis, detoxification after acetaminophen poisoning, adult respiratory distress syndrome (ARDS), hyperoxia-induced pulmonary damage, HIV infection, cancer, and heart disease [1-4]. Free radicals are critical in the determination of protein structure, regulation of enzyme activity, protein phosphorylation, and control of transcription factor activity and binding and NAC is often used to explore their role in these effects. However, despite its frequent use and the enormous clinical knowledge about this drug, free radical scavenging is often assumed to be the mechanism by which it brings about its effects although its exact targets are unknown. For example, it is unclear how NAC acts to down-regulate expression of the transcription factor NF-κB, which is a major mediator of inflammatory responses and controls expression of a large variety of genes encoding cytokines, growth factors, acute phase proteins, and cell adhesion and immunoregulatory molecules [5, 6].

The findings that NAC prevents NF-κB activation in response to a variety of signals, including TNF-α and ionizing radiation [7, 8] are generally taken as support for the involvement of free radicals in the process. TNF-α is thought to mediate both signal transduction and its cytotoxic effects through reactive oxygen intermediates (ROI). However, there is evidence that the lipoxygenase pathway may be more important than free radicals in mediating the latter [9]. Furthermore, some of the effects of NAC, like the G1-arrest described by Sekharam and

colleagues [10], are not easily explained through a simple mechanism involving direct scavenging of free radicals.

NF-κB is a family of homo- or hetero-dimers of proteins of the RelA/NF-κB family. They pre-exist in the cytosol, bound to inhibitor molecules (IκB) that prevent nuclear translocation of the complex. Classically, upon appropriate signaling, IκB is phosphorylated at two serine sites (ser32, ser36) by specific kinases (IKK), poly-ubiquitinated and degraded by the 26s proteasome. This releases NF-κB and allows nuclear translocation followed by initiation of transcription of dependent genes (for a review see [11]). Degradation of IκB by the 26s proteasome is a mandatory step for NF-κB activation in response to most signals. The 26s proteasome is a large protease of 2MDa that consists of a cylindrical 20s core particle formed by 4 rings each with seven alpha and beta subunits. The inner two rings form the catalytic site of the complex. These exhibit 5 distinct cleavage activities [12]. Activity is regulated over a wide range by substitution of constitutive beta-subunits by interferon-inducible subunits LMP2, LMP7 and MECL-1 [13, 14] and by 19s regulatory and 11s activator units that control substrate access, de-ubiquitination, and substrate linearization [15].

ATP- and ubiquitin-dependent protein degradation by the 26s proteasome is one of the most important degradation pathways of mammalian cells. The rate of degradation, as well as the rate of synthesis, regulates intracellular levels of proteins like p53, IκB, cJun, cFos and cyclins A, B and E, p21 and p27 [16, 17]. It therefore controls cellular responses in many physiological and pathophysiological conditions [12]. It plays an additional important role in the

immune system by determining the peptides that are expressed on the cell surface in association with MHC class I molecules [18-20]. Similarities between many of the effects attributed to NAC and those that follow inhibition of the ubiquitin-proteasome pathway led us to investigate a possible direct effect of NAC on 26s proteasome function using a well-established *in vitro* model for inflammatory responses. Our findings highlight an additional, and possibly major, pharmacological aspect of this frequently used drug.

Material & Methods

Cell culture

Cultures of ECV 304 human bladder carcinoma cells (DSMZ, Braunschweig, Germany), SiHa cervical carcinoma cells (ATCC) and RAW 264.7 murine macrophages (a generous gift of Dr. Guido Hildebrandt, Department of Radiation Oncology, University Leipzig) were grown in 75 cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2. ECV 304 cells are a variant of the T-24 bladder carcinoma [21], it exhibits many endothelial characteristics [22], and is often used as a model for inflammatory responses [23-25], because it's response to inflammatory stimuli is reminiscent of that of endothelial cells. It shows constitutive activation of the NF-κB signal transduction pathway [26]. The media used was DMEM (Gibco BRL) supplemented with 10 % FCS, 1 % penicillin/streptomycin (Gibco BRL).

Transfection

ECV 304 cells were maintained in DMEM (10 % FCS, 1% penicillin/streptomycin). 12 hours before transfection cells were trypsinized and plated at a density of 250.000 cells/well into six-well plates. Cells were transfected with 5 µg of a plasmid (pEGFP-N1, Clontech) coding for an ubiquitin-R-GFP fusion protein under control of a CMV promoter [27] (a kind gift from Dr. M. Masucci, Karolinska Institute, Sweden) using the Superfect transfection kit (Qiagen) and following the manufacturer's instructions. Transfected cells were maintained in DMEM (10 % FCS, 1% penicillin/streptomycin) supplemented with

500µg/ml G418 (Sigma) and clones were obtained. Expression of Ub-R-GFP was analyzed by flow cytometry (FL1-H, *FACSCalibur*, Becton Dickinson) using *CellQuest* Software before and after treatment with the proteasome inhibitor MG-132 (50 µM, Calbiochem) for 10 hours at 37° C. Clone #10 (ECV 304/10), which showed low background and high expression of Ub-R-GFP after MG-132 treatment, was used for inhibition experiments.

Drug treatment

NAC (Sigma) was dissolved in phenol-red-free PBS. The pH of the NAC solution was adjusted to pH 7.5. In proteasome function assays, the drug was added to the reaction mixture immediately before measurements were started. In gel shift experiments, media was removed 60 minutes prior to irradiation and replaced by phenol-red-free PBS supplemented with the drug or carrier.

Proteasome function assays

Proteasome function was measured as described previously [28] with some minor modifications. Briefly, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation. Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1000g for 5 minutes and 10.000g for 20 minutes. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard.

20 µg protein of each sample was diluted with buffer I to a final volume of 200 µl. The fluorogenic proteasome substrates SucLLVY-MCA (chymotrypsin-like, Sigma), Z-Leu-Leu-Leu-AMC (Calbiochem) and Boc-Val-Leu-Lys-AMC (trypsin-like, Sigma) were dissolved in DMSO and added in a final concentration of 80 µM in (0.8 % DMSO). Proteolytic activity was monitored continuously by the release of the fluorescent group 7-amido-4-methylcoumarin (AMC) measured in a fluorescence plate reader (Spectrafluor, Tecan and Spectra Max Gemini XS, Molecular Devices, 37° C) at 380/460 nm. In control experiments using lactacystin (10µM) and MG-132 the chymotryptic activity of the lysates was always inhibited by more than 90%, indicating that the observed cleavage activity was mainly based on proteasome function.

Irradiation

Cells were plated into Petri dishes. After 24 hours the cells were pre-incubated with NAC for 60 minutes. Cells were irradiated at room temperature using a ^{137}Cs-laboratory irradiator (JL Shephard, Mark I, dose rate of 5.80 Gy/min and IBL 637, CIS bio international, dose rate of 0.78 Gy/min). Corresponding controls were sham irradiated.

Electrophoretic mobility shift assays

Cells were dislodged mechanically, washed with ice-cold PBS, and lysed in TOTEX-buffer (20mM HEPES (pH 7.9), 0.35mM NaCl, 20% glycerol, 1% NP-40, 0.5 mM EDTA, 0.1mM EGTA, 0.5mM DTT, PMSF and aprotinin) for 30 minutes

on ice. Lysates were centrifuged at 12.000g for 5 minutes. Protein concentration was determined using the BCA protocol (Pierce) with BSA (Sigma) as standard. 15 µg protein of the resulting supernatant was incubated for 25 minutes at room temperature with 2 µl BSA (10 µg/µl), 2 µl dIdC (1 µg/µl), 4µl Ficoll-buffer (20% Ficoll 400, 100mM HEPES, 300 mM KCl, 10 mM DTT, 0.1mM PMSF), 2 µl buffer D+ (20mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% NP-40, 2 mM DTT, 0.1mM PMSF) and 1 µl of the [γ^{32}P]-ATP labeled oligonucleotide (Promega, NF-κB: AGT TGA GGG GAC TTT CCC AGG). For negative controls, unlabeled oligonucleotide was added in 50-fold excess. Gel analysis was carried out in native 4% acrylamide/0.5 % TBE gels. Dried gels were placed on a phosphor imaging screen, which was analyzed 24 hours later (Storm 860, Molecular Dynamics and IPR 1500, Fuji).

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 % Nonidet P-40, SDS, 1% protease inhibitor cocktail (P8340, Sigma)). Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin as standard. 20 µg of protein were subjected to SDS gel electrophoresis (0.1% SDS/7.5% polyacrylamide) and blotted to PVDF membranes. After blocking with 5% skim milk in PBS, Equity of loading was confirmed using a monoclonal antibody against α–tubulin (mouse anti human, CP06, 1:20.000, Oncogene). Membranes were stripped and incubated with a monoclonal antibody against human p53 (mouse anti human, OP43, 0.1µg/ml,

Oncogene), a polyclonal antibody against murine and human IκBα (rabbit anti-mouse, 554135, 1:20.000, BD) and a monoclonal murine antibody against poly-ubiquitinated proteins (1:5.000, clone FK2, Affinity). Secondary HRP-conjugated antibodies (rabbit anti-mouse (Dianova) and goat anti-rabbit (DAKO), 1:20.000) and the ECL Plus System (Amersham) were used for visualization.

Results

NAC is a free radical scavenger that has been shown to prevent NF-κB activation in response to a variety of stimuli [7, 29-31]. We confirmed this in ECV 304 and RAW 264.7 cells, using a gel shift assay. As shown in Fig. 1, NF-κB was activated in ECV 304 (A) and RAW 264.7 (B) cells 1 hour after irradiation with 30Gy. Pretreatment of cells with NAC (0, 7.5, 15 and 30 mM) for 60 minutes inhibited radiation-induced NF-κB activity in ECV 304 and RAW 264.7 cells in dose-dependent manner. The activation of NF-κB by ionizing radiation in RAW 264.7 macrophages was restricted to an increase of free p50/p65 heterodimers. Pre-incubation of RAW 264.7 cells with NAC prevented this induction but left constitutive p50/p50 homodimer activity unchanged. Similar inhibition could be achieved by using the proteasome inhibitor MG-132 (Fig 1C).

To investigate a possible relationship between NAC and proteasome function, we examined its effects on the chymotryptic cleavage activity of the 20s and 26s proteasome in ECV 304 cells. An assay previously published by Glas and colleagues [28] was used to monitor proteasome activity. This assay utilizes fluorogenic peptides to measure the specific cleavage activities of the 26s proteasome. Addition of NAC at concentration of 0, 0.5, 1, 2, 4, 7.5, 15 and 30 mM to proteasome extracts from ECV 304 cells resulted in a dose-dependent inhibition (r^2 = 0.85) of chymotrypsin-like 26s proteasome activity. At 30 mM NAC inhibited chymotryptic activity to 43.9 ± 1.2 % (mean ± standard deviation, $p<0.01$, two-sided t-test) of baseline cleavage rates (closed symbols, Fig.2A) and tryptic- and L3-cleavage activity of the 26s proteasome to 80.2 ± 5.1 % and 34.8

± 0.5 % (mean ± standard deviation) (Fig. 2B). 20s proteasome activity can be monitored by performing the assay in the presence of 0.03% SDS and in the absence of ATP [32]. NAC did not decrease cleavage activity under these conditions, but rather slightly increased it at higher concentrations (open symbols, Fig. 2A).

To confirm the effects of NAC of proteasome inhibition, ECV304/10 cells stably transfected with an expression plasmid for an Ub-R-GFP fusion protein were used. Under standard conditions the GFP protein is rapidly degraded by the 26s proteasome. After inhibition of proteasome function GFP accumulates in up to 20 % of the cells [27]. Incubation with NAC (15 mM) for 24 hours caused an increase of the GFP-positive fraction from initially 9.6 in untreated to 17.1% in NAC-treated cells (Fig. 2C) with a 1.3fold overall increase of the mean fluorescence.

Experiments were performed to confirm that the effects of NAC could be extended beyond NF-kB activation to include p53. SiHa cells express the HPV E6 protein, which mediates degradation of p53 by the ubiquitin/26s proteasome pathway. Total cellular protein extracts from SiHa cervical carcinoma cells were incubated with NAC (0, 15, 30 and 60 mM) for 20 hours and analyzed by western blotting using an antibody against p53, IκBα and poly-ubiquitin. Incubation with NAC caused an increase of p53 and IκBα protein levels and accumulation of poly-ubiqitinated proteins consistent with inhibition of the ubiquitin/26s proteasome pathway (Fig 2D and 2E).

Discussion

Most previous studies on NAC have focused on its anti-oxidative effects. It was shown to prevent activation of NF-κB induced by TNF-α and ionizing radiation [8, 30]. Both treatments are known to induce the formation of ROI and generation of these radicals could be prevented by NAC treatment. Together, these results have been taken to imply a role for ROI in NF-κB activation in response to TNF-α or radiation [33]. However, the exact mechanism of TNF-α-induced ROI formation is still not clear, nor is the link between ROI and NF-κB activation [34, 35] or the cytotoxic action of TNF-α. Indeed, a direct role for ROI in mediating TNF-α-related cytotoxicity was recently seriously questioned [9].

The classical pathway for activation of NF-κB involves IκB-kinases that phosphorylate IκB at two serine sides, which leads to polyubiquitination and subsequent degradation of IκB by the 26s proteasome. It is this degradation that releases the NF-κB dimer from its inhibitor IκB and allows NF-κB translocation into the nucleus to initiate gene transcription [11]. NF-κB dependent genes are thought to be required for radiation-induced inflammatory responses that could play roles in both tumor control and normal tissue damage after radiotherapy [36].

Inhibition of 26s proteasome function has been shown to prevent TNF-α-induced activation of NF-κB [37]. In agreement with the findings of Hallahan and coworkers [8], we have recently shown that radiation-induced activation of NF-κB is dependent on 26s proteasome function since it was prevented by the specific

proteasome inhibitor MG-132 [38], although an alternate pathway of radiation- and hypoxia-induced NF-κB activation has been described [39, 40].

In this study radiation-induced NF-κB activation was clearly prevented by pre-treatment of RAW264.7 cells with NAC. Furthermore NAC had a direct effect on proteasome function at a concentration that was optimal for prevention of NF-κB activation in response to TNF-α treatment [7]. NAC acted as an inhibitor of 26s chymotryptic, as well as L3- and, to a lesser extent, trypsin-like, proteasome activity, but failed to decrease 20s chymotryptic-like activity. Attempts to generate Lineweaver-Burk diagrams using 10, 20, 40 and 80μM concentrations of the fluorogenic substrate failed to identify a classical inhibition type. Together these observations give rise to the assumption that NAC targets one or more subunits of the 19s regulatory unit rather than interfering with the ß-subunits of the 20s core unit which are the catalytic sites of this protease complex. Our observation that NAC treatment causes accumulation of p53 in human cervical cancer cells confirmed an earlier report of an interference of NAC with this pathway [41]. Additionally, NAC treatment led to accumulation of IκBα, a ubiquitin/GFP fusion protein that is rapidly degraded by the 26s proteasome in untreated control cells, and poly-ubiquitinated proteins in general. This supports the conclusion that NAC targets the proteasome directly. In addition to providing a possible mechanism for the inhibitory effects of NAC on NF-κB activation, inhibition of proteasome function easily explains the G1-arrest observed after NAC-treatment [10] as a functional proteasome is required for G1/S transition [42].

The data presented in this study suggest that cellular responses to NAC treatment result, at least in part, from 26s proteasome inhibition, although our experimental setting did not allow determination of the contribution of radical scavenging, as opposed to proteasome inhibition, to the total observed effect.

Some previous studies have questioned the role of ROI scavenging in mediating the effects of NAC. For example, it has been pointed out that H_2O_2 is not rapidly scavenged by NAC [43]. This study even reported that H_2O_2 could be generated as a result of the auto-oxidation process of NAC in the presence of O_2. Previous studies have shown that proteasome function can be inhibited by H_2O_2 [32, 44] treatment and ionizing radiation [38]. However, the major site of these inhibitory effects appears to be the 20s core unit of the proteasome, rather than the 19s regulatory unit, which appears to be the site of action of NAC. This suggests that the proteasome is a highly redox-sensitive structure and that distinct structures may be the target for different chemical species.

Steps in this proteolytic degradation pathway, other than those mediated by the 19s regulatory subunit, might also be affected by *in vivo* treatment of cells with NAC, or other scavengers. For example, a necessary step for 26s proteasome dependent proteolysis is the tagging of proteins with ubiquitin, a 76-residue polypeptide involving a three-enzyme cascade. Ubiquitin is activated when its carboxy-terminal glycine is transformed into a high-energy thiol-ester intermediate by the ubiquitin-activating enzyme E1. The ubiquitin-carrier protein E2 transfers ubiquitin to a ubiquitin-protein ligase, E3, that catalyzes the conjugation of the activated carboxy-terminal ubiquitin to ε-amino groups of lysine

residues of the targeted protein (reviewed in [16]). Intracellular reduced GSH is required for E1 and E2 enzymes to form ubiquitin thiol-esters [45] and is critical for the process of ubiquitination. NAC increases the level of GSH in cells [46] and this could affect the degradation process. Therefore, both stimulatory and inhibitory effects of NAC on degradation rates would seem possible. Use of the Ub-GFP construct and the fluorogenic assay bypasses any stimulatory effects, but inhibition must predominate if the end result is NF-κB inhibition or p53 activation. This balance may not, however, be the same under all conditions.

Finally, this study raises a cautionary note regarding the use of free radical scavengers to implicate ROI in signaling events following exposure of cells to ionizing radiation and other signals. The proteasomal degradation pathway should be considered as an alternative to direct free radical scavenging as a target for the observed effects.

References

[1] Domenighetti, G., Quattropani, C., and Schaller, M. D. [Therapeutic use of N-acetylcysteine in acute lung diseases]. Rev Mal Respir, ***16:*** 29-37, 1999.

[2] Gibbs, B. F., Schmutzler, W., Vollrath, I. B., Brosthardt, P., Braam, U., Wolff, H. H., and Zwadlo-Klarwasser, G. Ambroxol inhibits the release of histamine, leukotrienes and cytokines from human leukocytes and mast cells. Inflamm Res, ***48:*** 86-93, 1999.

[3] Kelly, G. S. Clinical applications of N-acetylcysteine. Altern Med Rev, ***3:*** 114-127, 1998.

[4] Pizzulli, L., Hagendorff, A., Zirbes, M., Jung, W., and Luderitz, B. N-acetylcysteine attenuates nitroglycerin tolerance in patients with angina pectoris and normal left ventricular function. Am J Cardiol, ***79:*** 28-33, 1997.

[5] May, M. J. and Ghosh, S. Signal transduction through NF-kappa B. Immunology Today, ***19:*** 80-88, 1998.

[6] Pahl, H. L. Activators and target genes of Rel/NF-kappaB transcription factors. Oncogene, ***18:*** 6853-6866, 1999.

[7] Rahman, A., Kefer, J., Bando, M., Niles, W. D., and Malik, A. B. E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. Am J Physiol, ***275:*** L533-544, 1998.

[8] Hallahan, D. E., Virudachalam, S., and Kuchibhotla, J. Nuclear factor kappaB dominant negative genetic constructs inhibit X-ray induction of cell adhesion molecules in the vascular endothelium. Cancer Research, ***58:*** 5484-5488, 1998.

[9] O'Donnell, V. B., Spycher, S., and Azzi, A. Involvement of oxidants and oxidant-generating enzyme(s) in tumour- necrosis-factor-alpha-mediated apoptosis: role for lipoxygenase pathway but not mitochondrial respiratory chain. Biochem J, ***310:*** 133-141, 1995.

[10] Sekharam, M., Trotti, A., Cunnick, J. M., and Wu, J. Suppression of fibroblast cell cycle progression in G1 phase by N- acetylcysteine. Toxicol Appl Pharmacol, ***149:*** 210-216, 1998.

[11] Baeuerle, P. A. and Baltimore, D. NF-kappa B: ten years after. Cell, ***87:*** 13-20, 1996.

[12] Hasselgren, P. O. and Fischer, J. E. The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. Ann Surg, ***225:*** 307-316, 1997.

[13] Tanaka, K. Molecular biology of proteasomes. Molecular Biology Reports, ***21:*** 21-26, 1995.

[14] Stohwasser, R. and Kloetzel, P. M. Cytokine induced changes in proteasome subunit composition are concentration dependent. Biological Chemistry, ***377:*** 571-577, 1996.

[15] Ferrell, K., Wilkinson, C. R., Dubiel, W., and Gordon, C. Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem Sci, ***25:*** 83-88, 2000.

[16] Rolfe, M., Chiu, M. I., and Pagano, M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. Journal of Molecular Medicine, ***75:*** 5-17, 1997.

[17] Weissman, A. M. Regulating protein degradation by ubiquitination. Immunology Today, ***18:*** 189-198, 1997.

[18] Realini, C. A. and Rechsteiner, M. C. Proposed role of a gamma-interferon inducible proteasome-regulator in antigen presentation. Advances in Experimental Medicine and Biology, ***389:*** 51-61, 1996.

[19] Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, ***78:*** 761-771, 1994.

[20] Belich, M. P. and Trowsdale, J. Proteasome and class I antigen processing and presentation. Molecular Biology Reports, ***21:*** 53-56, 1995.

[21] Dirks, W. G., MacLeod, R. A., and Drexler, H. G. ECV304 (endothelial) is really T24 (bladder carcinoma): cell line cross- contamination at source [letter]. In Vitro Cell Dev Biol Anim, ***35:*** 558-559, 1999.

[22] Takahashi, K., Sawasaki, Y., Hata, J., Mukai, K., and Goto, T. Spontaneous transformation and immortalization of human endothelial cells. In Vitro Cell Dev Biol, ***26:*** 265-274, 1990.

[23] Lejoly-Boisseau, H., Appriou, M., Seigneur, M., Pruvost, A., Tribouley-Duret, J., and Tribouley, J. Schistosoma mansoni: in vitro adhesion of parasite eggs to the vascular endothelium. Subsequent inhibition by a monoclonal antibody directed to a carbohydrate epitope. Exp Parasitol, ***91:*** 20-29, 1999.

[24] Haberland, A., Knaus, T., Zaitsev, S. V., Stahn, R., Mistry, A. R., Coutelle, C., Haller, H., and Bottger, M. Calcium ions as efficient cofactor of polycation-mediated gene transfer. Biochim Biophys Acta, ***1445:*** 21-30, 1999.

[25] Meilhac, O., Escargueil-Blanc, I., Thiers, J. C., Salvayre, R., and Negre-Salvayre, A. Bcl-2 alters the balance between apoptosis and necrosis, but does not prevent cell death induced by oxidized low density lipoproteins. Faseb J, ***13:*** 485-494, 1999.

[26] Pajonk, F., Pajonk, K., and McBride, W. Apoptosis and radisensitization of Hodgkin's cells by proteasome inhibition. Int.J.Radiat.Oncol, Biol, ***47:*** 1025-1032, 2000.

[27] Dantuma, N. P., Lindsten, K., Glas, R., Jellne, M., and Masucci, M. G. Short-lived green fluorescent proteins for quantifying ubiquitin/proteasome-dependent proteolysis in living cells. Nat Biotechnol, ***18:*** 538-543., 2000.

[28] Glas, R., Bogyo, M., McMaster, J. S., Gaczynska, M., and Ploegh, H. L. A proteolytic system that compensates for loss of proteasome function. Nature, ***392:*** 618-622, 1998.

[29] Marumo, T., Schini-Kerth, V. B., and Busse, R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. Diabetes, ***48:*** 1131-1137, 1999.

[30] Lee, R., Beauparlant, P., Elford, H., Ponka, P., and Hiscott, J. Selective inhibition of l kappaB alpha phosphorylation and HIV-1 LTR- directed gene expression by novel antioxidant compounds. Virology, ***234:*** 277-290, 1997.

[31] Baeuml, H., Behrends, U., Peter, R. U., Mueller, S., Kammerbauer, C., Caughman, S. W., and Degitz, K. Ionizing radiation induces, via generation of reactive oxygen intermediates, intercellular adhesion molecule-1 (ICAM-1) gene

transcription and NF kappa B-like binding activity in the ICAM-1 transcriptional regulatory region. Free Radical Research, ***27:*** 127-142, 1997.

[32] Reinheckel, T., Sitte, N., Ullrich, O., Kuckelkorn, U., Davies, K. J., and Grune, T. Comparative resistance of the 20S and 26S proteasome to oxidative stress. Biochemical Journal, ***335:*** 637-642, 1998.

[33] Schreck, R., Albermann, K., and Baeuerle, P. A. Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). Free Radic Res Commun, ***17:*** 221-237, 1992.

[34] Larrick, J. W. and Wright, S. C. Cytotoxic mechanism of tumor necrosis factor-alpha. Faseb J, ***4:*** 3215-3223, 1990.

[35] Buttke, T. M. and Sandstrom, P. A. Oxidative stress as a mediator of apoptosis [see comments]. Immunol Today, ***15:*** 7-10, 1994.

[36] Hallahan, D., Kuchibhotla, J., and Wyble, C. Cell adhesion molecules mediate radiation-induced leukocyte adhesion to the vascular endothelium. Cancer Res, ***56:*** 5150-5155, 1996.

[37] Lum, R. T., Kerwar, S. S., Meyer, S. M., Nelson, M. G., Schow, S. R., Shiffman, D., Wick, M. M., and Joly, A. A new structural class of proteasome inhibitors that prevent NF-kappa B activation. Biochem Pharmacol, ***55:*** 1391-1397, 1998.

[38] Pajonk, F. and McBride, W. H. Ionizing radiation affects 26s proteasome function and associated molecular responses, even at low doses. Radiother Oncol, ***59:*** 203-212., 2001.

[39] Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B., Mueller-Dieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A., and Peyron, J. F. Tyrosine phosphorylation of I kappa B-alpha activates NF-kappa B without proteolytic degradation of I kappa B-alpha. Cell, ***86:*** 787-798, 1996.

[40] Raju, U., Gumin, G. J., Noel, F., and Tofilon, P. J. IkappaBalpha degradation is not a requirement for the X-ray-induced activation of nuclear factor kappaB in normal rat astrocytes and human brain tumour cells. International Journal of Radiation Biology, ***74:*** 617-624, 1998.

[41] Liu, M., Pelling, J. C., Ju, J., Chu, E., and Brash, D. E. Antioxidant action via p53-mediated apoptosis. Cancer Res, ***58:*** 1723-1729, 1998.

[42] Machiels, B. M., Henfling, M. E., Gerards, W. L., Broers, J. L., Bloemendal, H., Ramaekers, F. C., and Schutte, B. Detailed analysis of cell cycle kinetics upon proteasome inhibition. Cytometry, ***28:*** 243-252, 1997.

[43] Tuttle, S., Horan, A. M., Koch, C. J., Held, K., Manevich, Y., and Biaglow, J. Radiation-sensitive tyrosine phosphorylation of cellular proteins: sensitive to changes in GSH content induced by pretreatment with N- acetyl-L-cysteine or L-buthionine-S,R-sulfoximine. Int J Radiat Oncol Biol Phys, ***42:*** 833-838, 1998.

[44] Reinheckel, T., Ullrich, O., Sitte, N., and Grune, T. Differential impairment of 20S and 26S proteasome activities in human hematopoietic K562 cells during oxidative stress. Arch Biochem Biophys, ***377:*** 65-68, 2000.

[45] Obin, M., Shang, F., Gong, X., Handelman, G., Blumberg, J., and Taylor, A. Redox regulation of ubiquitin-conjugating enzymes: mechanistic insights using the thiol-specific oxidant diamide. Faseb J, ***12:*** 561-569, 1998.

[46] Wong, B. K., Chan, H. C., and Corcoran, G. B. Selective effects of N-acetylcysteine stereoisomers on hepatic glutathione and plasma sulfate in mice. Toxicol Appl Pharmacol, ***86:*** 421-429, 1986.

Figure legends

Figure 1

Gel shift experiments using 15µg of cytosolic extracts of ECV 304 (A) and RAW 264.7 (B) murine macrophages and a labeled double stranded oligonucleotide containing a consensus binding motif for NF-κB. Unlabeled oligonucleotide in 50fold molar excess was used as a negative control to demonstrate the specificity of the binding (lane 1 A, B and C). (A) Pre-treatment of ECV 304 cells with NAC for 1 hour decreased radiation-induced (30 Gy) NF-κB activation 1 hour after irradiation in a dose-dependent manner. Lane 1: negative control; lane 2: NAC 0 mM; lane 3: NAC 7.5 mM, lane 4: NAC 15 mM; lane 5: 30 mM. (B) Application of 30 Gy ionizing radiation caused an increase in p50/p65 heterodimer DNA-binding activity (lane 3) when compared to untreated controls (lane 2). This increase was prevented by pre-incubation with NAC in a dose-dependent manner (lane 4: 7.5 mM; lane 5: 15 mM; lane 6: 30 mM). In contrast DNA-binding activity of p50/p50 homodimers remained unchanged. (C) Pre-treatment of ECV 304 cells with MG-132 for 3 hours decreased radiation induced NF-κB activity. Lane 1: negative control; lane 2: control ; lane 3: 30 Gy, lane 4: 30 Gy MG-132 50 µM; lane 5: 30 Gy MG-132 25 µM.

Figure 2

(A) Effect of NAC on chymotryptic 20s (open symbols) and 26s (filled symbols) proteasome activity. Cleavage rate of SucLLVY-MCA (80µM) was assessed with

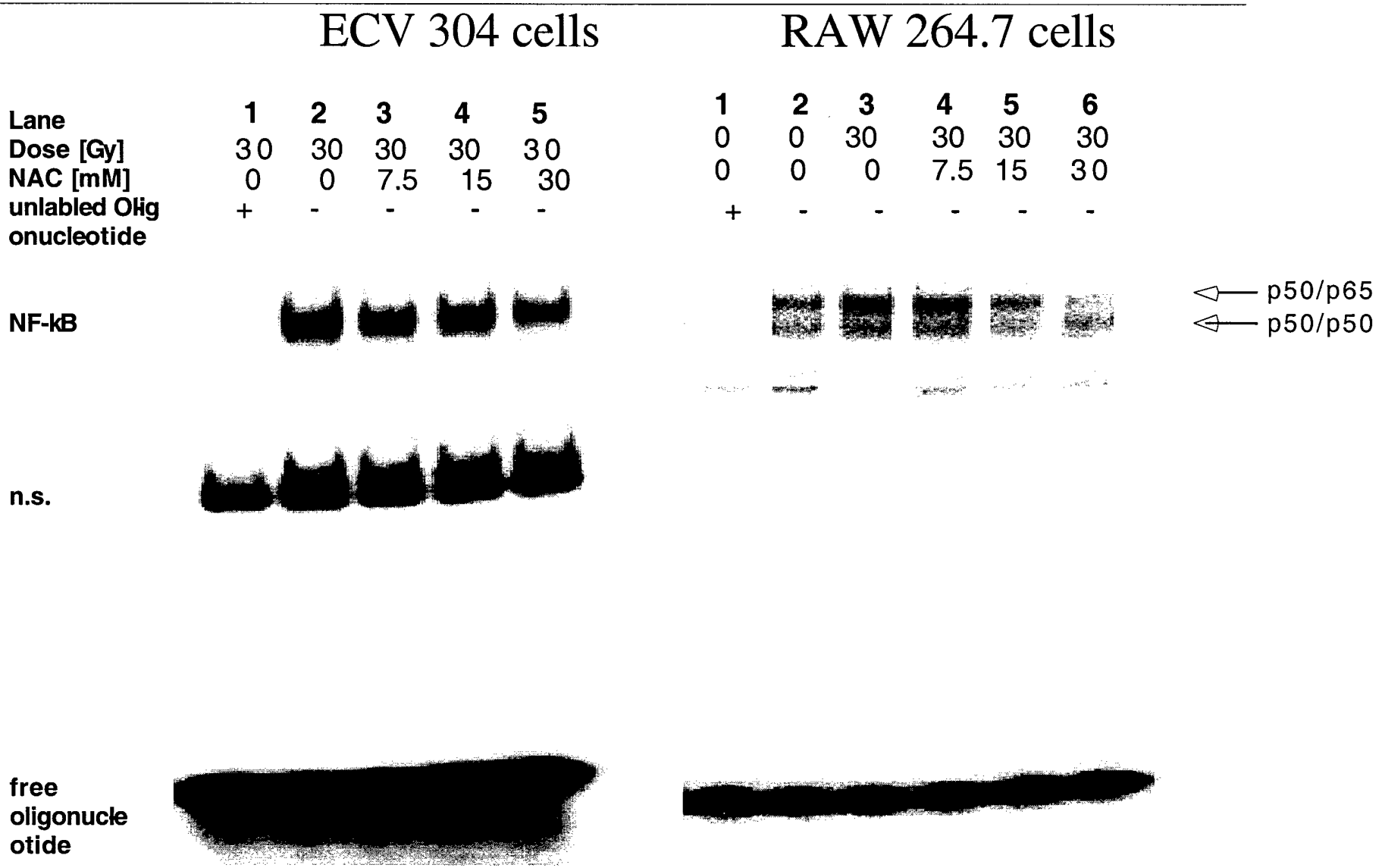
a fluorogenic peptide assay (excitation 380nM, emission 460 nm). 26s proteasome activity in crude extracts of ECV-304 cells was inhibited by N-acetyl-L-cysteine in a dose-dependent manner ((means from 4 measurements ± standard deviation) 0.5 mM NAC 94.5 % ± 5.6, 1 mM NAC 90 % ± 4.6, 2 mM: 85 %± 6.2, 4 mM 78.6 % ± 5.2, 7.5 mM 66.7 % ± 2.3, 15 mM: 54.6 % ± 2, 30 mM NAC: 43.9 % +/- 1.3, $p<0.001$ t-test). In contrast, 20s was not only unaffected by NAC treatment but seemed to be slightly increased at NAC concentrations of 4 mM and higher.

(B) Proteasome function assay using the fluorogenic proteasome substrates Z-Leu-Leu-Leu-AMC (L3-like) and Boc-Val-Leu-Lys-AMC (trypsin-like). L3- and tryptic-cleavage activity of the 26s proteasome were inhibited to 34.8 ± 0.5 % and 80.2 ± 5.1 % (mean ± standard deviation) of DMSO-treated controls, respectively, by 30mM NAC.

(C) Flowcytometric analysis of EVC 304 cells, stable transfected with an expression plasmid for a ubiquitin/GFP fusion protein. NAC treatment (15mM) for 24 hours increased the GFP-positive population of cells from initially 9.6 to 17.1%.

(D) and (E) Western blot analysis of SiHa cervical carcinoma cells treated with N-acetyl-L-cysteine (0, 15 and 30 mM) for 20h. A monoclonal antibody against α-tubulin was used to demonstrate equity of protein loading. Membrane was stripped and re-probed with antibodies against p53, IκBα and poly-ubiquitinated proteins. NAC treatment caused accumulation of p53, IκBα and poly-ubiquitinated proteins.

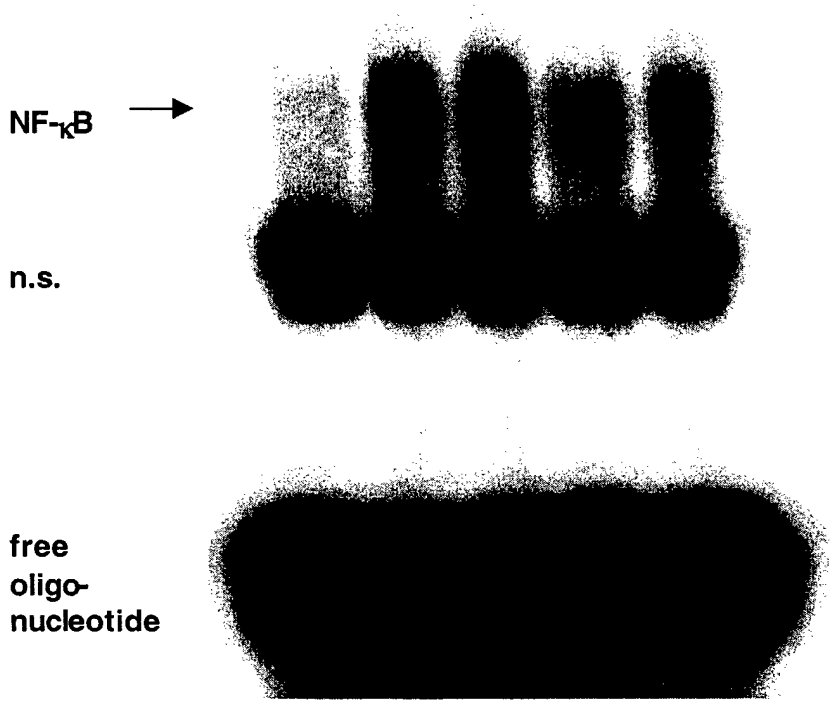
Effect of NAC on Radiation-Induced NF-κB Activity



Cells were pretreated with NAC for 1 hour before irradiation

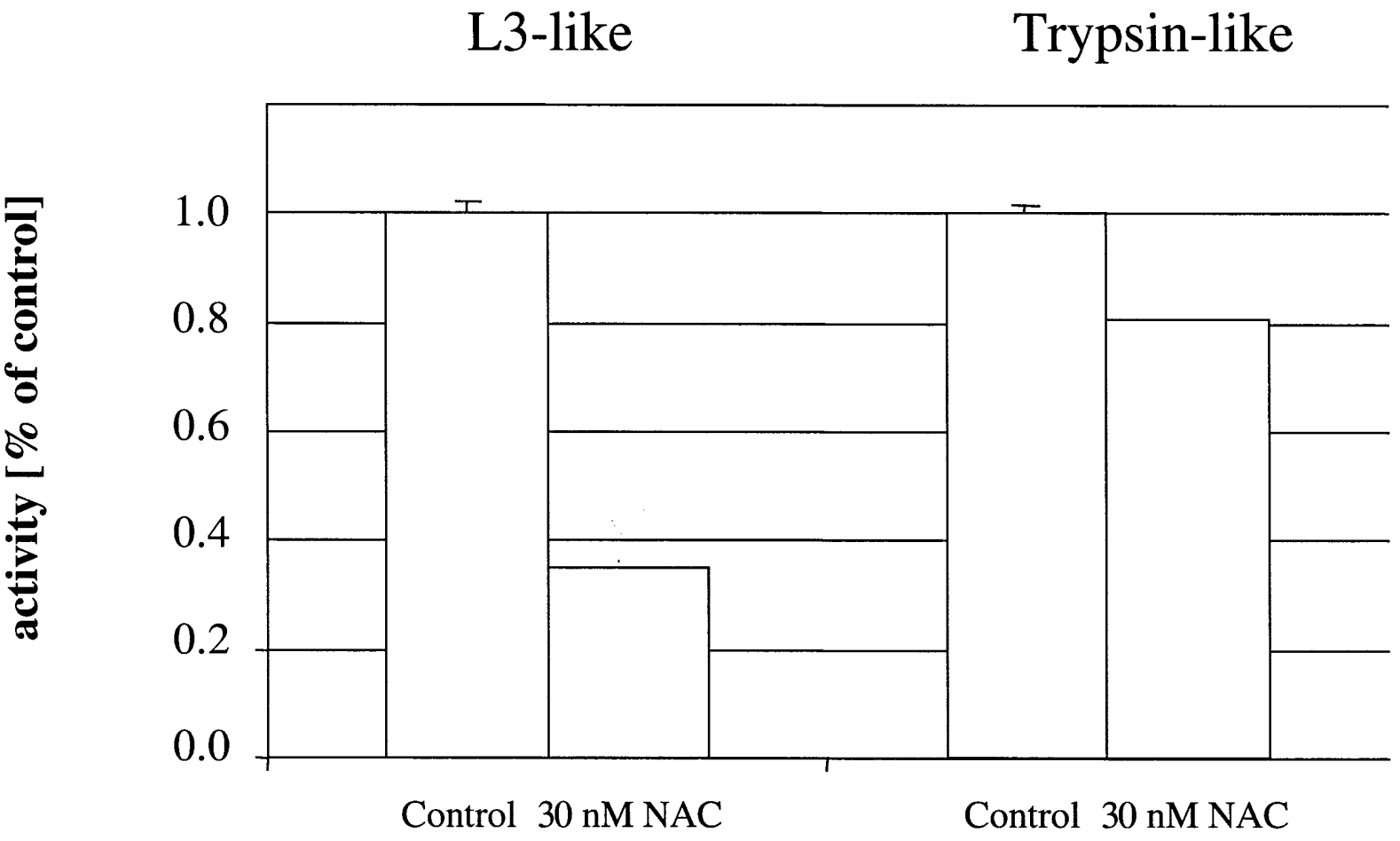
Effect of Proteasome Inhibition on NF-κB Expression

Lane	1	2	3	4	5
Dose [Gy]	30	0	30	30	30
MG-132 [μM]	0	0	0	50	25
unlabled Oligonuleotide	+	-	-	-	-

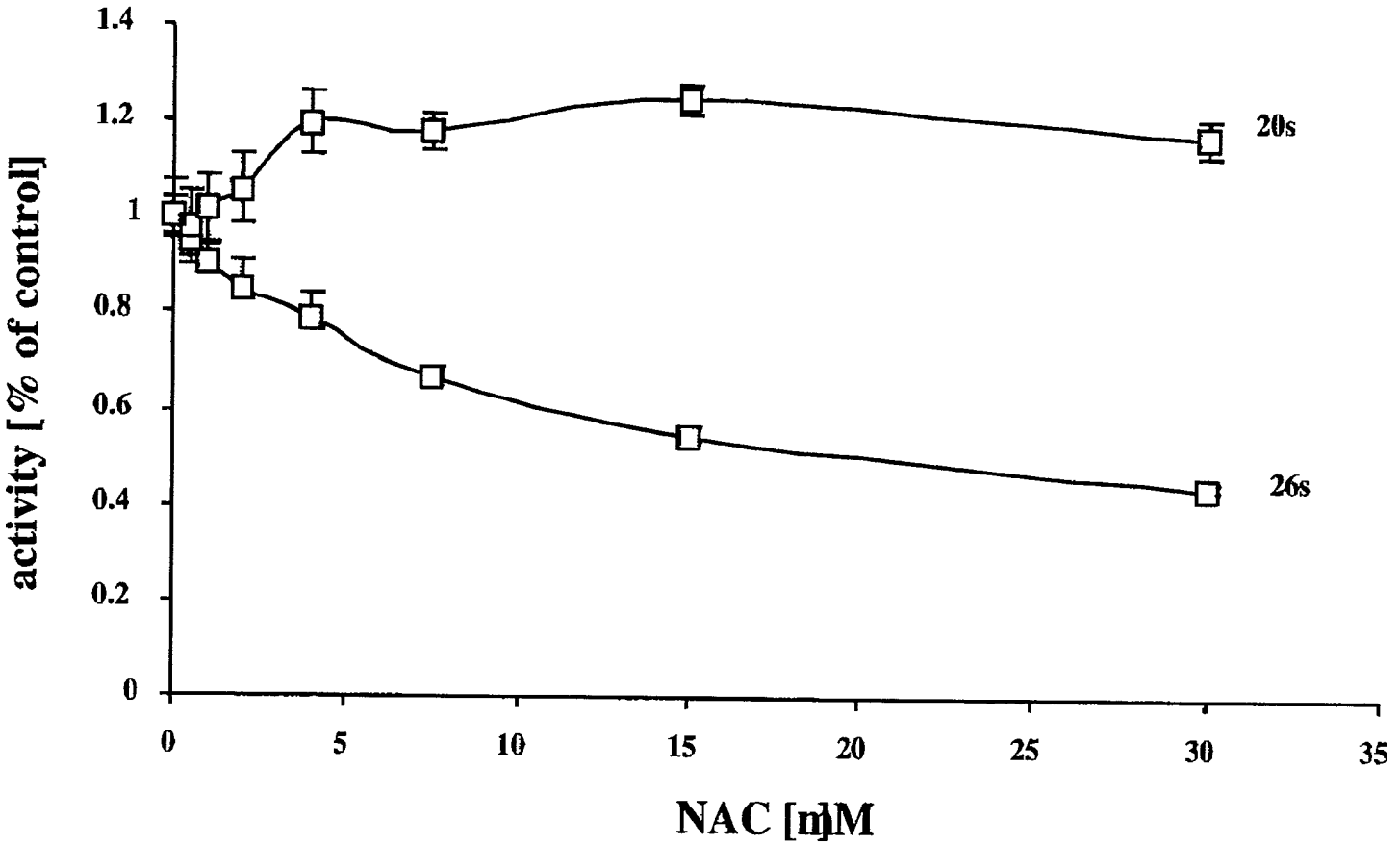


ECV304 cells were pretreated with MG132 for 3 hours and EMSA run for NF-kB

Effect of NAC on tryptic-like and L3-like Function of Proteasome (ECV304 cells)

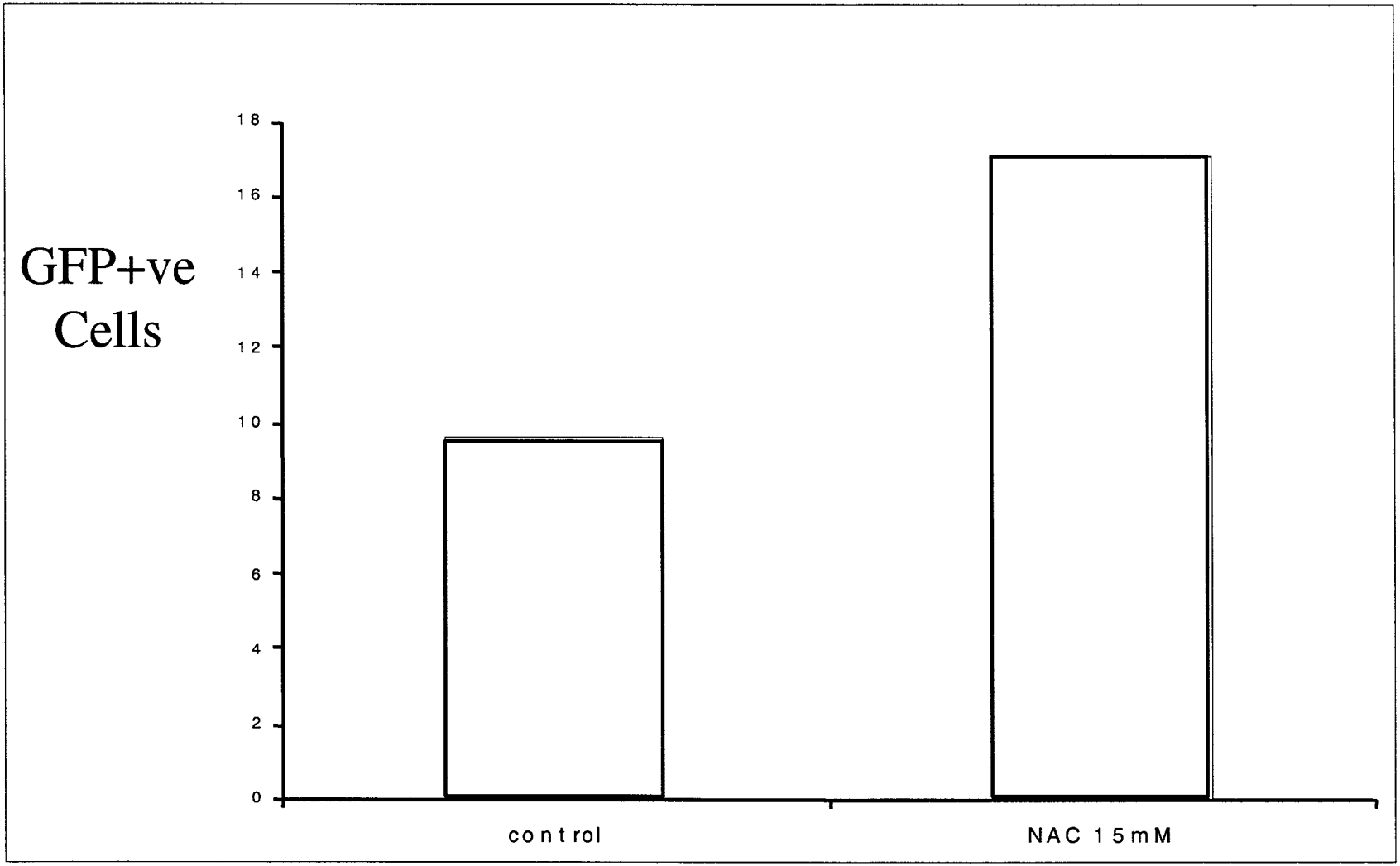


Effect of NAC on Chymotryptic-like Function of Proteasome (ECV304 cells)

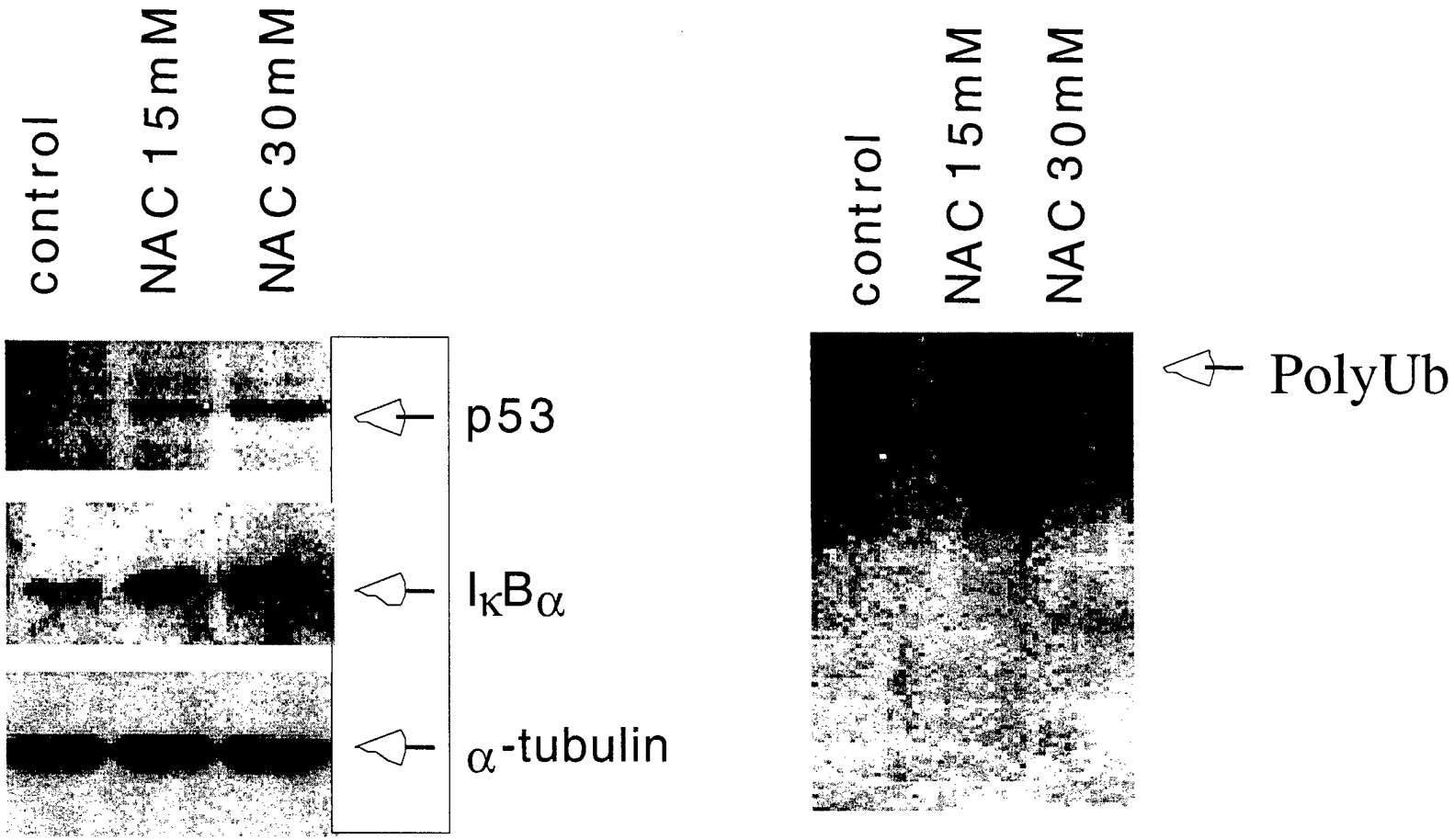


Cellular extracts were treated with NAC and proteasome activity measured by Suc LLVY-MCA cleavage

Effect of NAC on Ub-GFP Accumulation in Transfected ECV304 Cells



Effect of NAC on Gene Expression



SiHa cells were treated with NAC for 20 hrs and western blots performed

Hyperthermia treatment of prostate cancer cells, heat-induced proteasome inhibition and loss of androgen receptor expression

*¶Frank Pajonk[1], ¶Katrin Riess[1], ¶Alfred Sommer and ¶¶William H. McBride

¶ Department of Radiation Therapy, Radiological University Clinic Freiburg, Germany

¶¶ Department of Radiation Oncology, Experimental Division, UCLA School of Medicine

Running Title:

Keywords: Hyperthermia, androgen receptor, prostate cancer, radiation therapy

*Correspondence address: Department of Radiation Therapy, Radiological University Clinic, Hugstetter Str. 55, 79106 Freiburg i. Brsg. Germany; Tel.: +49-761-270-3862, Fax: +49-761-270-3982; email: pajonk@uni-freiburg.de

Footnotes: F.P. was supported by grants of the DFG (Deutsche Forschungsgemeinschaft) Pa 723/2-1 and Pa 723/3-1. K.R. was supported by a grant of the Boehringer Ingelheim Fonds. [1]These authors contribute equally to this work.

Abstract

Introduction

In western countries prostate carcinoma is the most common form of cancer and the second leading cause of death in males (*1*). While early forms of prostate cancer respond well to surgery or radiotherapy, patients with more advanced tumor stages will relapse and are usually not treated with curative intent. A significant increase in disease-free survival can been achieved by combination of surgery or radiotherapy with androgen-ablative treatment. Continuation of androgen-ablative treatment may delay recurrence in those that fail, but it is not curative as tumors become androgen-independent. The exact mechanisms of progression to an independent state are unclear. However, there is evidence (ref) that mutation of the androgen receptor (AR) gene and p53 mutations can be early events in prostate cancer carcinogenesis. Selection of this preexisting tumor cell population, as opposed to adaptation to hormone withdrawal, may therefore be possible. In any event, loss of androgen dependence appears to be associated with increased resistance to radiotherapy and chemotherapy, more aggressive behavior, and poor prognosis (Tso et al 2000). In order to solve these problems there is an ongoing research effort aimed at identifying new and effective forms of treatment for prostate cancer (*1*).

Hyperthermia is the oldest documented tumor treatment modality. Numerous *in-vitro* studies have shown the dose- and temperature-dependent radiosensitizing effect on cancer cells (reviewed in (*2*)). Although clinical studies with hyperthermia have yielded variable results, this can often be attributed to variations in the heating method, poor standardization of patients, and other uncontrolled variables. A major clinical problem that prevents hyperthermia from entering standard treatment regimens is the lack of a reliable method for real-time thermo-dosimetry. This is mainly because normal and tumor

vascularization cause a highly dynamic efflux of heat, making thermo-dosimetry extremely complex and at present impossible to predict in real time. In spite of these problems, some studies have shown encouraging results. For example, the combination of heat and irradiation has been reported to increase the number of complete responses up to 6 fold when compared to radiation alone (*3*), although the mechanism by which hyperthermia operates remains unclear.

A tumor entity that might allow comparatively easy clinical application of hyperthermia is carcinoma of the prostate. Because of its limited tumor diameters and vascularization, relatively superficial tumor location, and easy transurethral, transrectal, or transperianal accessibility, prostate cancer has become a major target of hyperthermia, although knowledge about clinical outcome and side effects is still poor. At present it is not possible to predict which patient will benefit from hyperthermia and pre-clinical studies are necessary to understand the molecular mechanisms that might determine response.

It has been recently reported that prostate cancer cells in general show elevated constitutive DNA-binding activity of the transcription factor NF-κB. NF-κB has been reported to be a negative regulator of androgen receptor expression. Also, we and others have demonstrated that inhibition of NF-κB induces apoptosis in prostate cancer cells (*4*). NF-κB is a hetero- or homodimer of the subunits p50, p52, p65/RelA, c-Rel, and Rel-B. It is sequestered preformed in the cytosol by inhibitor molecules of the IκB family (IκBα, IκBβ, IκBγ, Bcl-3, p100, and p105). Activation of this pathway is normally achieved by phosphorylation of one of the most important inhibitors, IκBα, at two serine-sites (Ser-32 and Ser-36) by IκB kinases. This marks IκBα for polyubiquitination and subsequent degradation by the 26s proteasome. Degradation of IκBα frees NF-κB for translocation

to the nucleus and activation of its target genetic programs (reviewed in (*5*)). The 26s proteasome is a protease of 2MDa responsible for the controlled ATP-and ubiquitin-dependent degradation of all short-lived (*6*) and 70-90% of all long-lived proteins (*6, 7*), including key molecules in signal transduction, cell cycle control, and immune responses (*8*). We hypothesized that hyperthermia might affect 26s proteasome activity, with consequent changes in NF-κB signal transduction and in tumor cell survival, radiosensitivity, and androgen dependency.

Material & Methods

Cell Culture

Cultures of PC-3 (ECACC), DU-145, and LnCaP (DMSZ, Braunschweig) human prostate carcinoma cells were grown in 75-cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2/95% air. Dulbecco's modified Eagle and RPMI 1640 medium (Cell Concepts, Freiburg) (what for what cell line?) were used supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (Gibco BRL).

Heat-shock treatment

For hyperthermia treatment, 5x10e6 cells were plated into Petri dishes. After overnight incubation at 37°C, dishes were sealed with parafilm and placed into an incubator preheated to 44°C for 1 hour before being returned to 37°C for the indicated times.

Irradiation and Clonogenic Assays

Hyperthermia treated (1 hour at 44°C) and control cells (1 hour at 37°C) were trypsinized, counted, and diluted to a final concentration of 10^6 cells/ml. The cell suspensions were immediately irradiated at room temperature with a ^{137}Cs laboratory irradiator (IBL 637, CIS Bio International) at a dose rate of 77.5 cGy /minute. Corresponding controls were sham irradiated. Colony forming assays were performed immediately after irradiation by plating an appropriate number of cells into culture dishes, in triplicate. After 14 days, cells were fixed and stained with 1% crystal violet, and the number of colonies containing more than 50 cells were counted. The surviving

fraction was normalized to the surviving fraction of the corresponding control, and survival curves were fitted by use of a linear-quadratic model.

Cell Extraction and Electrophoretic Mobility Shift Assay for NF-κB

For preparation of total cellular extracts, normal and treated cells were dislodged mechanically, washed with ice-cold phosphate-buffered saline (PBS), and lysed in TOTEX buffer (20 mM HEPES [pH 7.9], 0.35 mM NaCl, 20% glycerol, 1% Nonidet P-40, 0.5 mM EDTA, 0.1 mM EGTA, 0.5 mM dithiothreitol [DTT], 50μM phenylmethylsulfonyl fluoride [PMSF], and 90 trypsin inhibitor units [TIU's]/ml aprotinin) for 30 minutes on ice. The lysates were centrifuged at 12,000 x *g* for 5 minutes. Protein concentration was determined with the BCA protocol (Pierce). Fifteen micrograms of protein from the resulting supernatant was incubated for 25 minutes at room temperature with 2 μl of bovine serum albumin (10 μg/μl), 2 μl of dIdC (1 μg/μl), 4μl of Ficoll buffer (20% Ficoll 400, 100 mM HEPES, 300 mM KCl, 10 mM DTT, and 0.1 mM PMSF), 2 μl of buffer D+ (20 mM HEPES, 20% glycerol, 100 mM KCl, 0.5 mM EDTA, 0.25% Nonidet P-40, 2 mM DTT, and 0.1 mM PMSF), and 1 μl of [γ-^{32}P] ATP-labeled oligonucleotide (Promega, NF-κB: AGTTGAGGGGACTTTCCCAGG). For a negative control, unlabeled oligonucleotide was added to 50-fold excess. Gel analysis was carried out in native 4% polyacrylamide/0.5 fold TBE (Tris/Boric acid/EDTA) gels. Dried gels were placed on a phosphor screen for 24 hours and analyzed on a phosphor imager (Storm 860, Molecular Dynamics).

Immunoblotting

Cells were lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 % Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodium vanadate). Protein concentrations were determined using the BCA protocol (Pierce) with BSA (Sigma) as standard. 50 µg of protein were electrophoresed in a SDS gel (0.1% SDS/10% polyacrylamide) and blotted to PVDF membranes at 4° C. After blocking with Blotto-buffer (Tris-buffered saline, 0.1% Tween 20, 5% skim milk) for one hour at room temperature the membranes were incubated with a polyclonal antibody against murine IκBα (0.5µg/ml, Invitrogen) or a monoclonal antibody against the human androgen receptor (Pharmingen, 2µg/ml) for one hour at room temperature. A secondary HRP-conjugated goat-anti-mouse antibody (Serotec, 1:10.000) and the ECLplus system (Amersham) were used for visualization. Protein levels were normalized to expression of α-tubulin (Oncogene).

Proteasome function assays

Proteasome function was measured as described previously (*9*), with some minor modifications. To obtain crude cellular extracts, cells were washed with PBS, then with buffer I (50 mM Tris, pH 7.4, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP), and pelleted by centrifugation (1000g, 5 minutes, 4° C). Glass beads and homogenization buffer (50 mM Tris, pH 7.4, 1 mM DTT, 5 mM $MgCl_2$, 2 mM ATP, 250 mM sucrose) were added and cells were vortexed for 1 minute. Beads and cell debris were removed by centrifugation at 1000g for 5 minutes and 10.000g for 20 minutes at 4°C. Protein concentration was determined by the Micro BCA protocol (Pierce) with BSA (Sigma) as standard. To measure 26s proteasome activity, 20 µg protein of crude cellular extracts of each sample was diluted with buffer I to a final volume of 200 µl. For assessment of 20s proteasome

activity, 20μg of protein was diluted to a final volume of 200μl in a buffer consisting of 50mM Tris/HCl, p7.9, 0.5mM EDTA, and 0.05% SDS. The fluorogenic proteasome substrate SucLLVY-MCA (chymotrypsin-like, Sigma) was dissolved in DMSO and added in a final concentration of 80 μM in 1% DMSO. Proteolytic activity was continuously monitored by measuring the release of the fluorescent group 7-amido-4-methylcoumarin (AMC) in a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, 37° C) at 380/460 nm.

Determination of Apoptosis

In addition to monitoring apoptosis using morphological criteria, apoptotic cells were detected with an 'In Situ Cell Death Kit' (Boeringer Mannheim). The manufacturer's protocol was followed with some minor modifications. Briefly, attached and detached cells were collected, centrifuged, fixed in ice-cold 75% ethanol, washed with PBS, and pelleted by centrifugation for 5 minutes at 500 x *g*. Cells were permeabilized by resuspension in a solution of 0.1 % Triton X-100 and 0.1% sodium citrate and incubation for 2 minutes on ice. Cells were washed twice in PBS, resuspended in TUNEL (terminal deoxynucleotidyltransferase-mediated UTP end labeling) reaction mixture, and incubated for 60 minutes at 37°C. After three washes with PBS, fluorescence was measured at 518 nm in a flow cytometer (FACScan, Becton Dickinson) and analyzed with the 'CellQuest' software (Becton Dickinson).

Results

Heat-shock down-regulates NF-κB DNA-binding activity

Hyperthermia has been reported to cause apoptosis in many cell lines including PC-3 prostate cancer cells (*10*). This observation was confirmed in our present study. Twenty four hours after 1 hour heat treatment at 44° C, PC-3 cells showed morphological signs of apoptosis with membrane blebbing and chromatin condensation that was confirmed by TUNEL staining (Fig. 1).

Constitutive activity of the anti-apoptotic transcription factor NF-κB is high in many prostate cancer cell lines, including PC-3 cells, and inhibition of NF-κB can induce apoptosis in such cells (*4*). We therefore hypothesized that the pro-apoptotic effect of hyperthermia on PC3 cells might be through alterations in the DNA-binding activity of this transcription factor. EMSA was used to examine NF-κB activity in extracts from cells 1.5 hours after completion of hyperthermia treatment (44^0C for 1 hour) and/or irradiation (20 Gy). PC3, DU-145, and LNCaP cell lines were used to allow more general conclusions to be drawn (Fig 2A, B, C). Although constitutive baseline levels of NF-κB activity differed between the cell lines, hyperthermia almost complete inhibited constitutive and radiation-induced NF-κB activity in all three cell lines. Inhibition was not prevented by blocking transcriptional activity with cyclohexamide (CHX, 25μg/ml, for 30 minutes), excluding induction of an endogenous inhibitor of NF-κB as a mechanism, although pretreatment of cells with the drug did elevate baseline and radiation-induced NF-κB activity (Fig. 2A, B, C). Western blotting of protein extracts from the same cells (AT THE SAME TIME POINT ??) revealed stabilization of IκBα by

hyperthermia treatment (Fig. 3D), indicating an inhibitory effect of heat either on IκB-kinase activity, ubiquitination of IκBα, or proteasome function.

Heat-shock impairs 26s proteasome function

Specific inhibitors of proteasome function have been shown to induce apoptosis in human prostate cancer cells. The mechanism remains unclear, but it has been reported to be independent of p53, the JNK kinase pathway, bcl-2 (*12*), Bcl-X(L), Bax, Bad, Bak, or cytochrome c (*13*). Since functional 26s proteasome activity is an obligatory precondition for activation of the NF-κB signal transduction pathway, we hypothesized that hyperthermia might affect NF-κB activation by alter 26s proteasome cleavage activity. Using a fluorogenic assay, heat-shock was found to decrease 26s proteasome activity to 36.2 ± 3% (PC-3), 33.4 ± 8.4% (DU-145) and 45 ± 3.4% (LnCaP), respectively in the 3 prostate cancer cell lines (Fig. 4). This effect was also observed when cells were pretreated with cyclohexamide (CHX, 25μg/ml for 30 minutes, data not shown). The function of the 26s proteasome is dependent on ubiquitin and ATP, which reflects activity in vivo, but proteolytic activity resides in the 20s core unit. This can be assessed by addition of SDS to the 26s complex. To determine whether the thermo-sensitive component of the 26s proteasome is in the 19s regulatory units or in the 20s core we repeated the experiments in the absence of ATP and the presence of SDS (0.03%). In all 3 cell lines, 20s activity was not significantly altered by hyperthermia treatment (data not shown).

Heat-shock down-regulates AR protein levels

NF-κB has been recently reported to be a negative regulator of androgen receptor expression (*14, 15*). Since heat-shock down-regulated NF-κB in all three cell lines, we tested if androgen receptor expression was increased by heat-shock. Cells were incubated for one hour at 44° C and thereafter at 37° C for an additional 90 minutes. Total cellular protein was separated by SDS-PAGE and blotted to PVDF membranes. Immunoblotting using a monoclonal antibody against human androgen receptor could not detect any AR expression in PC-3 and DU-145 cells. LnCaP cells in contrary showed strong expression of the human androgen receptor. Heat-shock treatment for one hour at 44° C did not cause any accumulation of AR protein in PC-3 and DU-145 cells, even up to 24 hours (not shown), but AR expression was completely abrogated in LnCaP cells 90 minutes after heat treatment (Fig. 5A). The extent of androgen receptor down-regulation in LnCaP cell was dependent on the duration of heat-shock treatment (Fig. 5B) and did not recover within 24 hours (data not shown). The observed effect was not prevented by inhibition of transcriptional activity, inhibition of proteasome function or of calpain I & II (Fig. 5C). To examine the involvement of proteases other than the proteasome and calpains we incubated LnCaP cells with a panel of protease inhibitors including the unspecific caspase inhibitor Z-VAD-FMK (20μM), PMSF (50μM), pepstatin (1.5μM), antipain (100μM) and aprotinin (1.5μM) 1 hour before and during heat-shock treatment. However, we could not prevent heat-induced disappearance of AR protein (Fig. 5D).

Discussion

Although the molecular basis of hyperthermia is poorly understood, it is frequently used alone or in combination with radiotherapy in the treatment of prostate cancer (*16-19*). In this study we investigated the effect of hyperthermia on proteasome function, NF-κB activity, and androgen receptor expression in human prostate cancer cell lines.

We recently demonstrated that human PC3 prostate cancer cells exhibit high constitutive expression of the anti-apoptotic transcription factor NF-κB (*4*). Inhibition of NF-κB by transduction with an IκB super-repressor gene induced apoptosis in PC-3 cells (*4*). Here we demonstrate that constitutive activity of NF-κB was also found in DU-145 and LnCaP human prostate cancer cells. Treatment of all three cell lines with heat-shock down-regulated constitutive and radiation-induced activation of NF-κB. Down-regulation of NF-κB after hyperthermia treatment has also been reported by other groups (*20,21*). While Curry and coworkers (*21*) observed an inhibition of the IκB-kinases, we found that hyperthermia inhibited 26s proteasome function, providing an alternative pathway for heat-induced NF-κB inhibition through blocking IkBα degradation. Impairment of proteasome function by heat is in agreement with the findings of Kuckelkorn and coworkers (*22*). Since 20s proteasome activity remained unchanged after heat-shock our data suggest that the thermo-sensitive component of the proteasome is located in the 19s regulatory unit rather than in the 20s core unit. Down-regulation of NF-κB DNA-binding activity by heat-induced proteasome inhibition offers a molecular mechanism by which hyperthermia could operate in cancer cells to induce apoptosis. Proteasome inhibition is known to induce apoptosis in most cancer cells (*12,13,23-27*) as well as sensitize surviving cells to ionizing radiation (*24,28*). The impact of NF-κB on the intrinsic

radiosensitivity of cancer cells is controversial (*4,28,29*). However, decreased proteasome function following heat treatment might also account for the well-established radiosensitizing effect of hyperthermia (ref).

Intriguingly, we also found that heat down-regulated AR expression in LNCaP cells. Survival of most tumor cells that originate from the prostate depends on the presence of androgen. Thus, prostate cancer is usually controlled by androgen ablation for many years. The appearance of tumor cell populations lacking androgen-dependence following androgen ablation is however inevitable and responsible for failure of this treatment. The mechanisms underlying development of androgen independence are unclear, but in some cases may be caused by down-regulation of AR expression or mutation of the AR gene combined with p53 mutations, which are thought to be early events during carcinogenesis occurring in a small subset of cancer cells (*30-32*). The expression of the androgen receptor is a tightly regulated process than involves recruitment of several transcription factors (*14,15,34*). Repression of androgen receptor gene expression occurs by binding of NF-κB to the promoter region of the AR gene (*15*). Heat-induced down-regulation of NF-κB might therefore be expected to result in increased AR protein expression in AR negative PC-3 cells, which are known to have minimal expression of functional androgen receptor mRNA and protein (*35,36*). However, we could not detect any increase in AR protein levels in PC-3 or DU-145 cells over a period of 24 hours after heat treatment. In contrast, LnCaP human prostate cancer cells that have wild type AR mRNA and protein levels (*37*) immediately down-regulated expression and did not recover over a period of 24 hours. The decrease in AR protein levels could not be prevented by inhibition of calpain I & II or inhibition of the 26s proteasome, underlining the independence of our

observation from these proteolytic pathways. This is at least in part contrary to results of an earlier study reporting ubiquitination and degradation of AR by the proteasomal pathway, which could be inhibited by MG-132 treatment (*38*). Hyperthermia is known to activate lysosomal enzyme activity (*39*), which is mainly based on cathepsins (*40*) and hydrolysis of the androgen receptor is mediated by cathepsin D (*41*). However, preincubation of LnCaP cells with a panel of protease inhibitors blocking cysteine proteases and chymotrypsin (PMSF,) cathepsin D (pepstatin), kallikrein, plasmin and trypsin (aprotinin), caspases (Z-VAD-FMK) and cathepsin A (Antipain) also failed to prevent heat-induced abrogation of AR protein, leaving the mechanism of heat-induced loss of androgen receptor elusive.

If the loss of AR in LnCaP cells reflects the behavior of androgen dependent prostate cancer cells *in vivo,* our study raises the question as to whether poorly performed clinical hyperthermia would drive the rapid acquisition of androgen independency, in particular in cells that have mutations affecting apoptotic pathways. Further studies are needed to explore the clinical significance of our findings.

Figure Legends

Fig.1 *Heat-induced apoptosis in PC-3 prostate cancer cells*

Flow-cytometric analysis of PC-3 prostate cancer cells. Incubation at 44°C for 1 hour leads to TUNEL-positive staining cells 24 hours after heat-shock (right panel). In contrast, no significant increase of the apoptotic fraction was observed after treatment at 42° C for 1 hour (left panel).

Fig. 2 *Hyperthermia treatment sensitizes DU-145 prostate cancer cells to ionizing radiation*

Clonogenic assay with DU-145 cells. Cells were irradiated at room temperature, plated into culture dishes in triplicate and subsequently treated with 37°C and 44°C for 1 hour. After 14 days cells were fixed with ethanol and stained with crystal violet. Colonies consisting of more than 50 cells were counted and normalized against the corresponding un-irradiated control. Hyperthermia treatment decreased the plating efficiency of DU-145 cells from 7.4% to 5.7% and sensitized DU-145 cells for ionizing radiation with a change in α/β from 2.4 to 1.03.

Fig. 3 *Hyperthermia downregulates constitutive and radiation-induced NF-κB activity*

Representative gel-shift experiments with cytosolic protein extracts from DU-145 **(A)**, PC-3 (B) and LnCaP **(C)** human prostate cancer cells. Constitutive NF-κB DNA-binding activity was found in all three cell lines (lanes 2). Irradiation with 20 Gy caused an increase of NF-κB DNA-binding activity after 90 minutes (lanes 4). Pre-incubation of the

cells with CHX (25μg/ml, 30 minutes before start of heat treatment) increased constitutive and radiation-induced NF-κB DNA-binding activity (lanes 6 and 8). Treatment of the cells with hyperthermia (1 hour at 44° C) abrogated constitutive and radiation induced NF-κB DNA-binding activity (lanes 3 and 5) independent of protein de-novo synthesis (lanes 7 and 9). Lanes 1: Negative control; unlabeled oligonucleotide was added in 50-fold molar excess to demonstrate the specificity of the binding reaction.

Fig. 4 *Hyperthermia inhibits 26s proteasome function*

Representative results of proteasome function assays for the chymotryptic cleavage activity of the fully functional regulated 26s proteasome. Release of the fluorescent group 7-amido-4-methylcoumarin from the proteasome substrate SucLLVY-MCA was continuously monitored in a fluorescence plate reader for 30 minutes (excitation/emission 380/460 nm, 37° C). Heat treatment of human prostate cancer cell lines led to inhibition of 26s proteasome function in PC-3, DU-145 and LnCaP prostate cancer cells 90 minutes after heat treatment. This effect was independent of protein de-novo synthesis and restricted to 26s proteasome function, while the activity of the 20s core unit remained unchanged.

Fig. 5 *Hyperthermia treatment abrogates expression of androgen receptor protein in androgen-dependent prostate cancer cells.*

(A) Western blot analysis of total cellular lysates from androgen receptor negative PC-3 (lanes 1 and 2), DU-145 (lanes 2 and 4) and androgen receptor positive LnCaP human prostate cancer cells (lane 5 and 6). Equity of loading was

confirmed using an anti-α-tubulin antibody (60 kDa). Heat treatment does not induce androgen receptor expression (110 kDa) in PC-3 and DU-145 cells. In contrast, LnCaP cells completely loose androgen receptor expression after treatment at 44° C for 1 hour.

(B) Western blot analysis of total cellular lysates from androgen receptor positive LnCaP human prostate cancer cells incubated 0 (lane 1), 15 (lane 2), 30 (lane 3), 45 (lane 4), 60 (lane 5), 75 (lane 6), 90 (lane 7) and 120 minutes (lane 8) at 44° C. Equity of loading was confirmed using an anti-α-tubulin antibody (60 kDa). Heat treatment causes a time-at-44 degree-dependent decrease of androgen receptor expression (110 kDa).

(C) Western blot analysis of total cellular lysates from androgen receptor positive LnCaP human prostate cancer cells. Equity of loading was confirmed using an anti-α-tubulin antibody (60 kDa). Constitutive expression of androgen receptor protein (110 kDa, lane 1) was completely abrogated by hyperthermia treatment (lane 2, 44° C for 1 hour). This effect could not be inhibted by preincubation with cyclohexamide (25μg/ml for 30 minutes, lanes 3 and 4) or MG-132 (50μM, 30 minutes, lanes 5 and 6) excluding gene induction in general as well as proteasomal or calpain-mediated proteolysis of the androgen receptor as a possible mechanism.

(D) Western blot analysis of total cellular lysates from androgen receptor positive LnCaP human prostate cancer cells. Equity of loading was confirmed using an anti-α-tubulin antibody (60 kDa). Cells were pretreated with PMSF (lane 3), pepstatin (lane 4), aprotinin (lane 5), Z-VAD-FMK (lane 6) and antipain (lane 7)

for one hour. Protease inhibition with non of the protease inhibitor used prevented heat-induced (lane 2) abrogation of constitutive androgen receptor expression (lane 1).

Literature

(*1*) Sweeney P, Pisters LL: Ad5CMVp53 gene therapy for locally advanced prostate cancer--where do we stand? World J Urol 2000;18:121-4.

(*2*) Kampinga HH, Dikomey E: Hyperthermic radiosensitization: mode of action and clinical relevance. Int J Radiat Biol 2001;77:399-408.

(*3*) Overgaard J: The current and potential role of hyperthermia in radiotherapy. Int J Radiat Oncol Biol Phys 1989;16:535-49.

(*4*) Pajonk F, Pajonk K, McBride WH: Inhibition of NF-kappaB, clonogenicity, and radiosensitivity of human cancer cells. J Natl Cancer Inst 1999;91:1956-60.

(*5*) Baeuerle PA, Baltimore D: NF-kappa B: ten years after. Cell 1996;87:13-20.

(*6*) Ciechanover A: The ubiquitin-proteasome proteolytic pathway. Cell 1994;79:13-21.

(*7*) Lee DH, Goldberg AL: Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in Saccharomyces cerevisiae. J Biol Chem 1996;271:27280-4.

(*8*) Rolfe M, Chiu MI, Pagano M: The ubiquitin-mediated proteolytic pathway as a therapeutic area. Journal of Molecular Medicine 1997;75:5-17.

(*9*) Glas R, Bogyo M, McMaster JS, Gaczynska M, Ploegh HL: A proteolytic system that compensates for loss of proteasome function. Nature 1998;392:618-22.

(*10*) Li WX, Franklin WA: Radiation- and heat-induced apoptosis in PC-3 prostate cancer cells. Radiat Res 1998;150:190-4.

(*11*) Leith JT: In vitro radiation sensitivity of the LNCaP prostatic tumor cell line. Prostate 1994;24:119-24.

(*12*) Herrmann JL, Briones F, Jr., Brisbay S, Logothetis CJ, McDonnell TJ: Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53. Oncogene 1998;17:2889-99.

(*13*) Kitagawa H, Tani E, Ikemoto H, Ozaki I, Nakano A, Omura S: Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. FEBS Lett 1999;443:181-6.

(*14*) Supakar PC, Roy AK: Role of transcription factors in the age-dependent regulation of the androgen receptor gene in rat liver. Biol Signals 1996;5:170-9.

(*15*) Supakar PC, Jung MH, Song CS, Chatterjee B, Roy AK: Nuclear factor kappa B functions as a negative regulator for the rat androgen receptor gene and NF-kappa B activity increases during the age- dependent desensitization of the liver. J Biol Chem 1995;270:837-42.

(*16*) Algan O, Fosmire H, Hynynen K, Dalkin B, Cui H, Drach G, et al: External beam radiotherapy and hyperthermia in the treatment of patients with locally advanced prostate carcinoma. Cancer 2000;89:399-403.

(*17*) Hurwitz MD, Kaplan ID, Svensson GK, Hynynen K, Hansen MS: Feasibility and patient tolerance of a novel transrectal ultrasound hyperthermia system for treatment of prostate cancer. Int J Hyperthermia 2001;17:31-7.

(*18*) Diederich CJ, Nau WH, Burdette EC, Bustany IS, Deardorff DL, Stauffer PR: Combination of transurethral and interstitial ultrasound applicators for high-temperature prostate thermal therapy. Int J Hyperthermia 2000;16:385-403.

(*19*) Tucker RD, Huidobro C, Larson T, Platz CE: Use of permanent interstitial temperature self-regulating rods for ablation of prostate cancer. J Endourol 2000;14:511-7.

(*20*) Pritts TA, Wang Q, Sun X, Moon MR, Fischer DR, Fischer JE, et al: Induction of the stress response in vivo decreases nuclear factor-kappa B activity in jejunal mucosa of endotoxemic mice. Arch Surg 2000;135:860-6.

(*21*) Curry HA, Clemens RA, Shah S, Bradbury CM, Botero A, Goswami P, et al: Heat shock inhibits radiation-induced activation of NF-kappaB via inhibition of I-kappaB kinase. J Biol Chem 1999;274:23061-7.

(*22*) Kuckelkorn U, Knuehl C, Boes-Fabian B, Drung I, Kloetzel PM: The effect of heat shock on 20S/26S proteasomes. Biol Chem 2000;381:1017-23.

(*23*) Zhang XM, Lin H, Chen C, Chen BD: Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochem J 1999;340:127-33.

(*24*) Pajonk F, Pajonk K, McBride W: Apoptosis and radisensitization of Hodgkin's cells by proteasome inhibition. Int.J.Radiat.Oncol, Biol 2000;47:1025-32.

(*25*) McDade TP, Perugini RA, Vittimberga FJ, Jr., Callery MP: Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. Surgery 1999;126:371-7.

(*26*) Adams J, Palombella VJ, Sausville EA, Johnson J, Destree A, Lazarus DD, et al: Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999;59:2615-22.

(*27*) An B, Goldfarb RH, Siman R, Dou QP: Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death Differ 1998;5:1062-75.

(*28*) Russo SM, Tepper JE, Baldwin AS, Jr., Liu R, Adams J, Elliott P, et al: Enhancement of radiosensitivity by proteasome inhibition: implications for a role of NF-kappaB. Int J Radiat Oncol Biol Phys 2001;50:183-93.

(*29*) Jung M, Zhang Y, Lee S, Dritschilo A: Correction of radiation sensitivity in ataxia telangiectasia cells by a truncated I kappa B-alpha. Science 1995;268:1619-21.

(*30*) Navone NM, Labate ME, Troncoso P, Pisters LL, Conti CJ, von Eschenbach AC, et al: p53 mutations in prostate cancer bone metastases suggest that selected p53 mutants in the primary site define foci with metastatic potential. J Urol 1999;161:304-8.

(*31*) Navone NM, Troncoso P, Pisters LL, Goodrow TL, Palmer JL, Nichols WW, et al: p53 protein accumulation and gene mutation in the progression of human prostate carcinoma. J Natl Cancer Inst 1993;85:1657-69.

(*32*) Marcelli M, Ittmann M, Mariani S, Sutherland R, Nigam R, Murthy L, et al: Androgen receptor mutations in prostate cancer. Cancer Res 2000;60:944-9.

(*33*) Dorkin TJ, Robson CN, Neal DE: The molecular pathology of urological malignancies. J Pathol 1997;183:380-7.

(*34*) Mora GR, Mahesh VB: Autoregulation of androgen receptor in rat ventral prostate: involvement of c-fos as a negative regulator. Mol Cell Endocrinol 1996;124:111-20.

(*35*) Lin MF, Meng TC, Rao PS, Chang C, Schonthal AH, Lin FF: Expression of human prostatic acid phosphatase correlates with androgen- stimulated cell proliferation in prostate cancer cell lines. J Biol Chem 1998;273:5939-47.

(*36*) Jarrard DF, Kinoshita H, Shi Y, Sandefur C, Hoff D, Meisner LF, et al: Methylation of the androgen receptor promoter CpG island is associated with loss of androgen receptor expression in prostate cancer cells. Cancer Res 1998;58:5310-4.

(*37*) Suzuki H, Sato N, Watabe Y, Masai M, Seino S, Shimazaki J: Androgen receptor gene mutations in human prostate cancer. J Steroid Biochem Mol Biol 1993;46:759-65.

(*38*) Sheflin L, Keegan B, Zhang W, Spaulding SW: Inhibiting proteasomes in human HepG2 and LNCaP cells increases endogenous androgen receptor levels. Biochem Biophys Res Commun 2000;276:144-50.

(*39*) Yamauchi N, Watanabe N, Maeda M, Okamoto T, Sasaki H, Tsuji N, et al: Mechanism of synergistic cytotoxic effect between tumor necrosis factor and hyperthermia. Jpn J Cancer Res 1992;83:540-5.

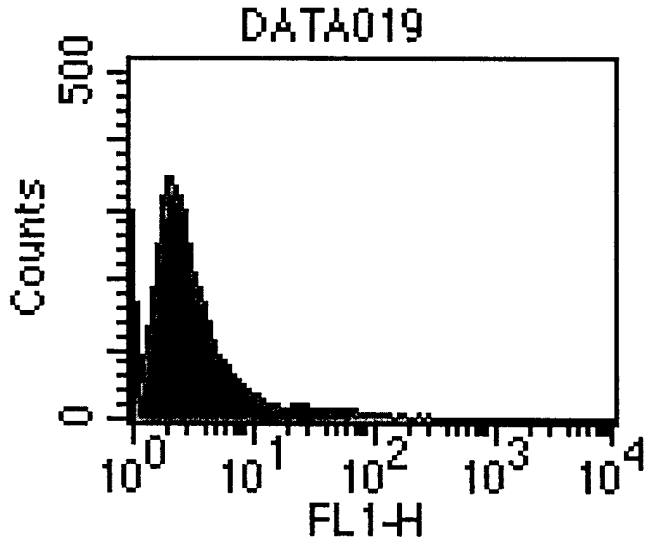
(*40*) Hasselgren PO, Fischer JE: The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. Ann Surg 1997;225:307-16.

(*41*) Mordente JA, Choudhury MS, Tazaki H, Mallouh C, Konno S: Hydrolysis of androgen receptor by cathepsin D: its biological significance in human prostate cancer. Br J Urol 1998;82:431-5.

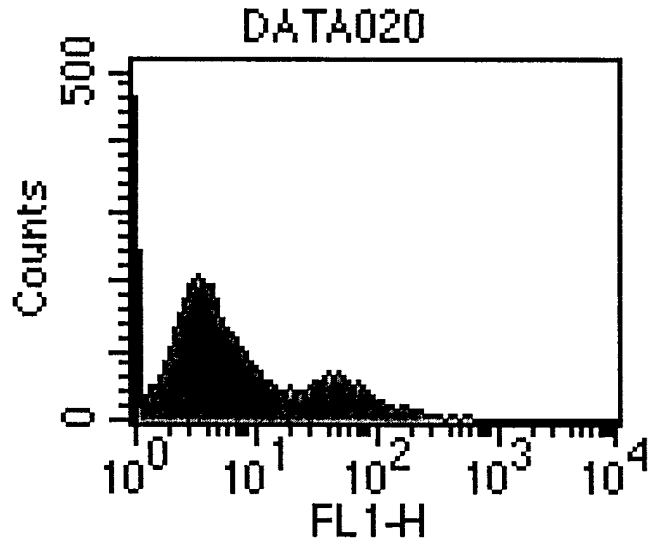
(*42*) Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, et al: Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 1996;379:88-91.

Heat-Induced Apoptosis in PC-3 cells

42 deg.

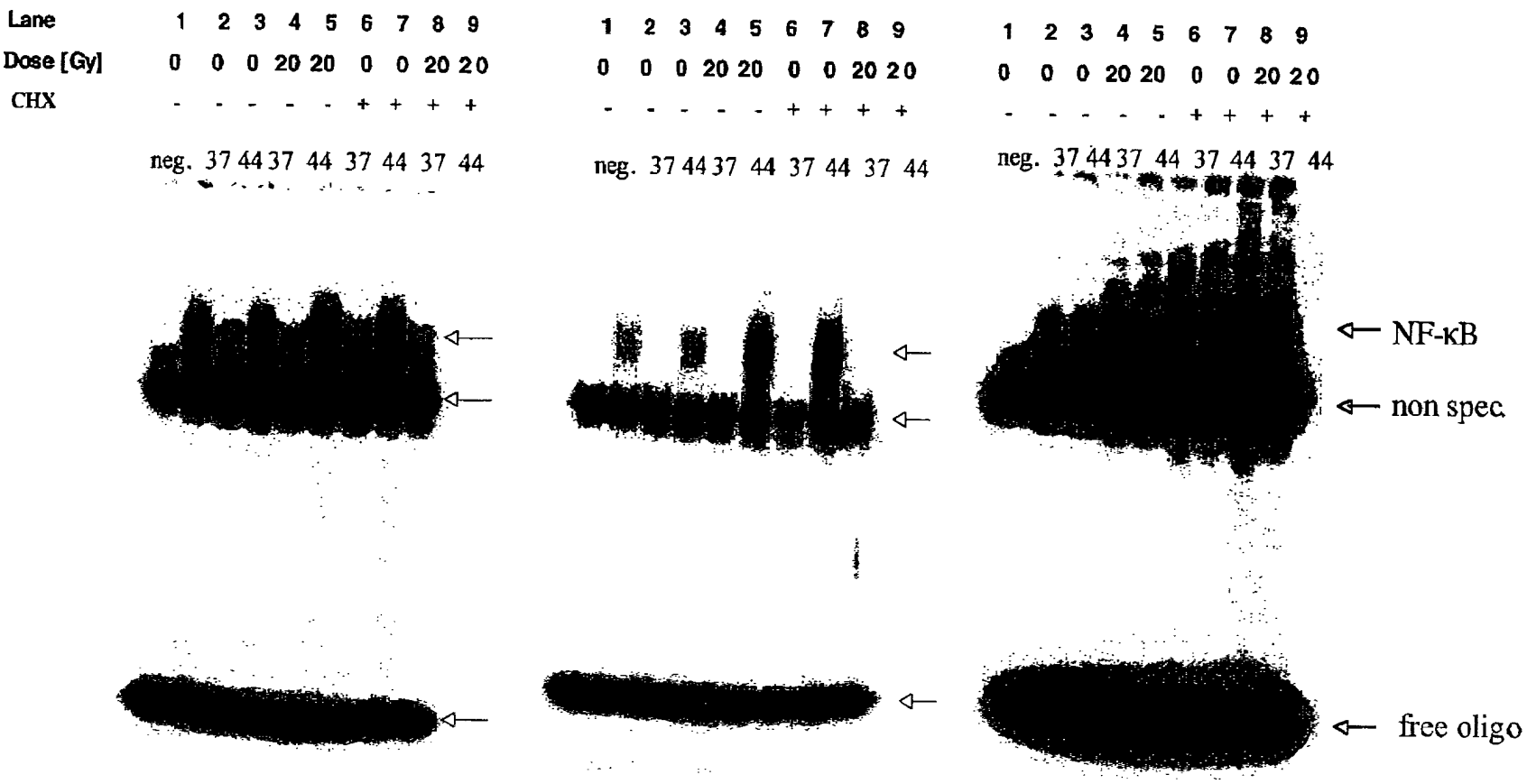


44 deg.



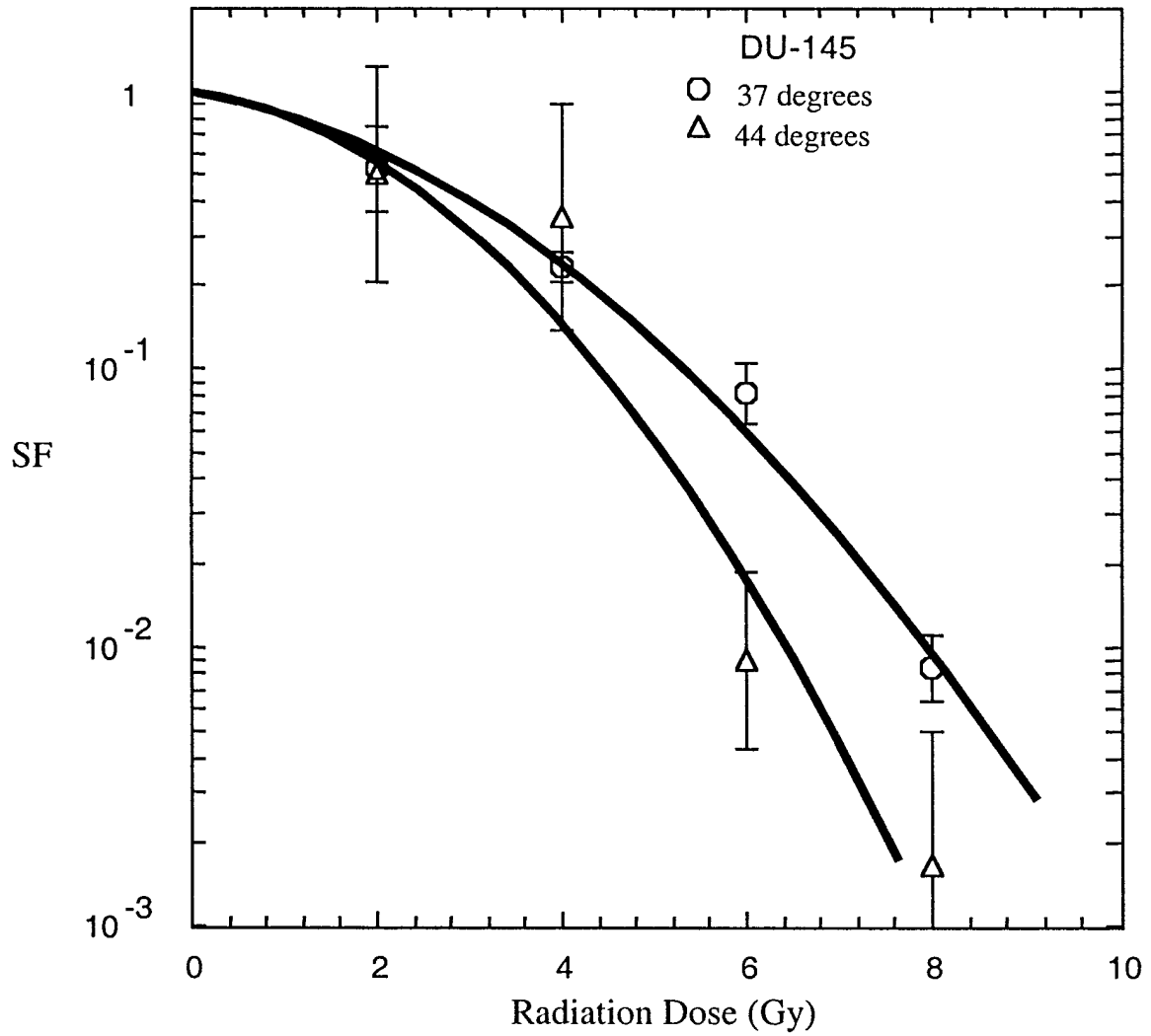
TUNEL staining 24 hours after heat application

Heat Blocks Constitutive and Radiation-Induced NF-kB in PC3, DU-145, and LNCaP Cells

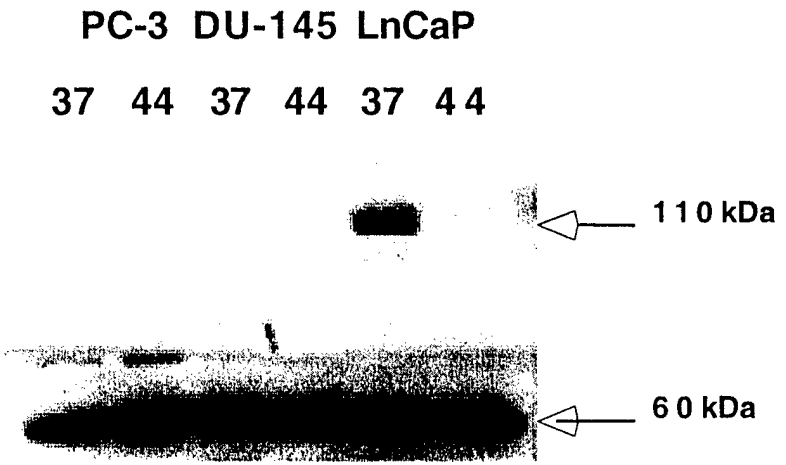


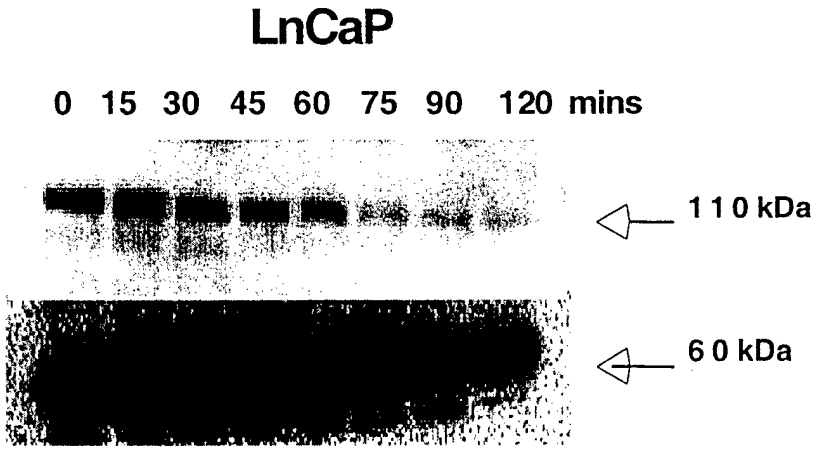
EMSA; heat for 1 hour at 44oC +/- cyclohexamide (25mg/ml) 30 mins)

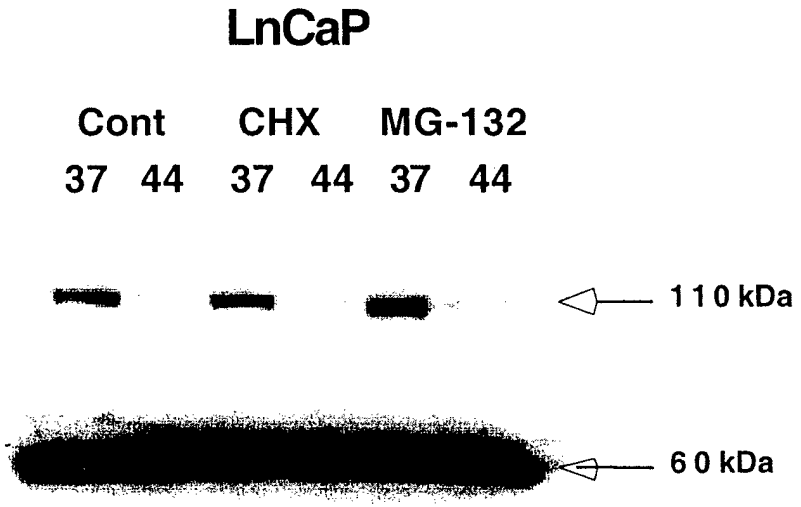
Heat Sensitizes DU-145 Cells to Irradiation

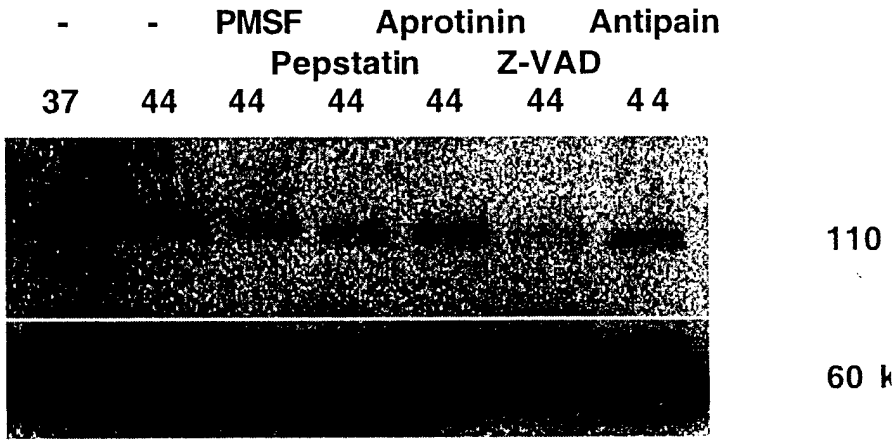


The Effect of Heat on Androgen Receptor Expression

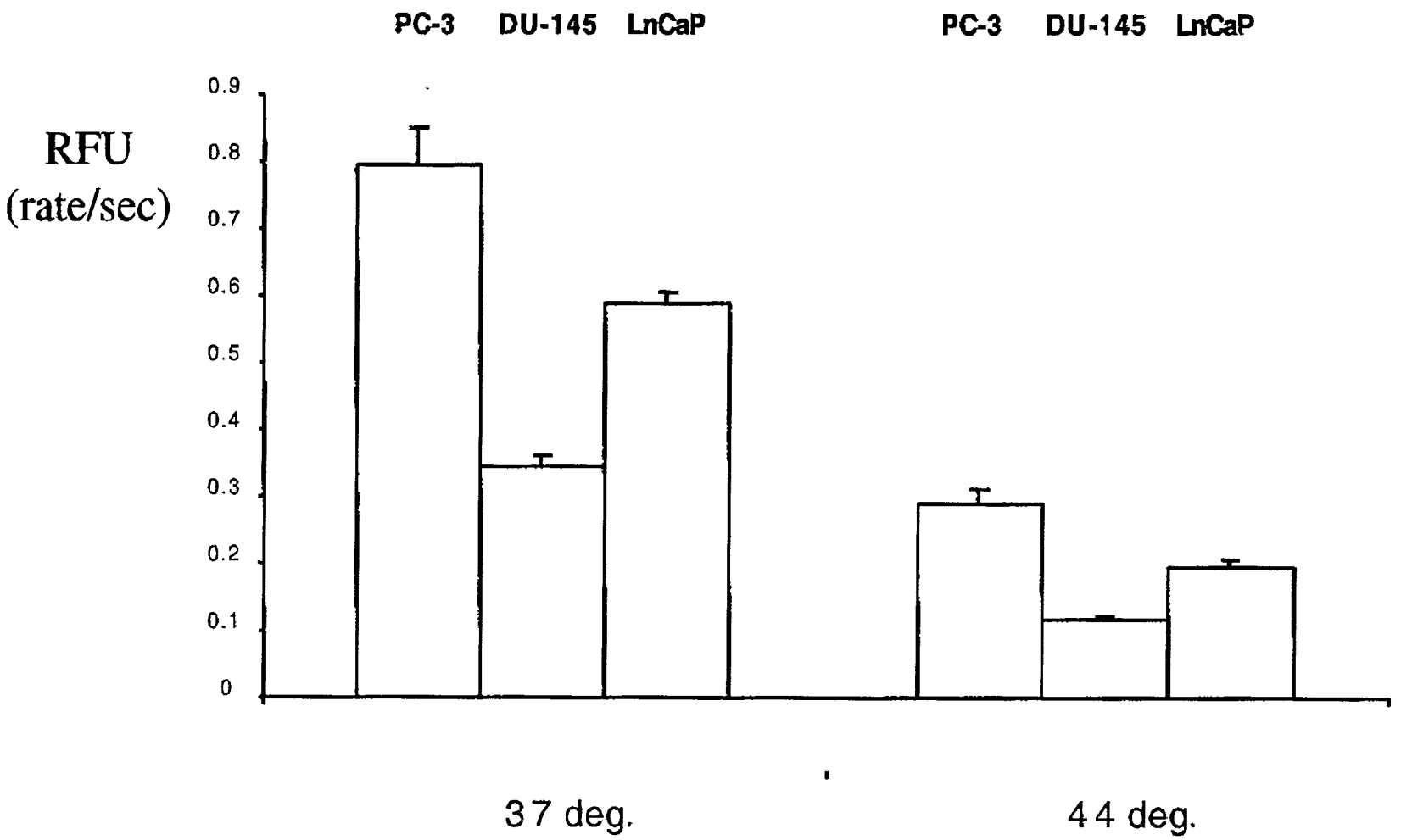








Heat Affects Chymotryptic 26s Proteasome Activity in Human Prostate Cancer Cells



Inhibition of 26s proteasome function sensitizes human cancer cells to ionizing irradiation by a DNA-PK-independent mechanism

Frank Pajonk and William H. McBride

Department of Radiation Oncology, Experimental Division, UCLA School of Medicine

Running Title: The proteasome inhibitor MG-132 is a radiosensitizer

Indexing phrases: prostate cancer, radiation response, 26s proteasome, MG-132, radiosensitizer

Correspondence address: Department of Radiation Oncology, Roy E. Coats Research Laboratories, UCLA School of Medicine, 10833 Le Conte Avenue, Los Angeles, CA 90095-1714; Tel.: (310) 825-8589, Fax: (310) 206-1260; email: fpajonk@ucla.edu

Includes:
5 copies
8 Figures

Abstract

Pajonk, F. and McBride, W.H. Inhibition of 26s proteasome function sensitizes human cancer cells to ionizing irradiation by a DNA-PK-independent mechanism. *Radiat. Res.*

The 26s proteasome plays a central role in the control of signal transduction, the cell cycle, cell death, and the immune response. Inhibition of its function leads to initiation of apoptosis in many cancer cell lines. Caspase-3, which is activated during apoptosis, can cleave DNA-PKcs, which is involved in DNA-double strand repair. This pathway could result in radiosensitization. In order investigate a possible impact of proteasome function on radiosensitivity, the human prostate cancer cell line PC-3 was treated with the proteasome inhibitor MG-132 in combination with ionizing radiation. Radiosensitivity was assessed with a clonogenic assay. Cell cycle distribution, apoptosis, caspase-3 activity, DNA-PKcs protein levels and DNA-PK activity were monitored. MG-132 induced apoptosis and sensitized PC-3 cells to ionizing radiation. Induction of apoptosis did not involve an early recruitment of caspase-3 and did not effect DNA-PKcs protein levels or DNA-PK activity. This study adds an additional aspect to the broad spectrum of 26s proteasome function and introduces proteasome inhibitors as a new class of radiosensitizers.

Introduction

One of the most challenging ongoing efforts in radiotherapy is the search for agents that utilize tumor specific characteristics in order to radiosensitize tumor cells without an increase in normal tissue complications. The mutations that are associated with the malignant phenotype are largely in molecules that control cell proliferation and/or cell death. The impact of these pathways on the response to radiotherapy is a subject of controversy. Radiosensitive tumors often have high proliferation and apoptotic indices (1, 2). Mutations that affect the functional integrity of the tumor suppressor gene p53 have been implicated as resulting in radioresistance (3). Also, in a number, but not all, tumor diseases, this mutation is associated with shorter disease free survival after chemotherapy and radiation therapy (4, 5, 6, 7). Modification of pathways involved in cell cycle control and apoptosis may, therefore, alter intrinsic cellular radiation responses.

The 26s proteasome is a large protease of 2MDa responsible for the controlled degradation of 70-90% of all cellular proteins (8) including cell cycle regulators and transcription factors like cyclin A, B and E, p21 and p27, p53, cJun, cFos. Additionally, it controls the NF-κB transduction pathway through degradation of IκB subsequent to serine phosphorylation and ubiquitination (9, 10). The proteasome therefore controls many of the cellular responses to stress signals, such as hypoxia/reperfusion, DNA damage, cytokines, hyperthermia, and oxidation that might induce cell cycle arrest, DNA repair, apoptosis, or prime cells for survival.

The 26s proteasome bears 5 different cleavage activity (11) and its function is highly regulated (12, 13, 14, 15, 16). Highly specific and potent inhibitors of the 26s proteasome like lactacystein have been shown to induce apoptosis in several cell lines (17, 18, 19,

20). In this study we used the proteasome inhibitor MG-132 to explore, in PC-3 prostate cancer cells, the impact of 26s proteasome function on the response to ionizing radiation and possible mechanisms for its effect.

Material & Methods

Cell culture

Cultures of PC3 human prostate carcinoma (ATCC) and HD-My-Z Hodgkin cells (DSMZ, Braunschweig) were grown in 75 cm^2 flasks (Falcon) at 37° C in a humidified atmosphere at 5 % CO_2. The medium used was DMEM medium (Gibco BRL) supplemented with 10 % FCS, 1 % penicillin/streptomycin (Gibco BRL), and 0.5 g/ml fungizone (amphotericin B, GibcoBRL).

MG-132 treatment

MG-132 (Calbiochem) was dissolved in DMSO (10mM) and small aliquots (30μl) were stored at –20° C. 3 hours before irradiation growth medium was replaced by medium containing MG-132 (50μM, 0.5% DMSO). Control cells were subjected to DMSO treatment alone (0.5%). Cells were incubated at 37°C for 3 hours, washed with DMEM, trypsinized, diluted and irradiated at room temperature.

Irradiation

Exponentially growing PC3 and HD-My-Z cells were trypsinized, counted, and diluted. The cell suspensions were immediately irradiated at room temperature using a ^{137}Cs-

laboratory irradiator (JL Shephard, Mark I) at a dose rate of 5.80 Gy/min. Corresponding controls were sham irradiated.

Clonogenic survival

Colony-forming assays with PC3 cells were performed immediately after irradiation by plating an appropriate number of cells into culture dishes, in triplicate. Viability was assessed by a dye exclusion test with trypan blue. After 14 days, colonies were fixed, stained with crystal violet, and were counted if they consisted of more than 50 cells. The fraction of cells surviving irradiation was normalized to the surviving fraction of the corresponding control and survival values and curves were fitted to the data using a linear-quadratic model. Data shown resulted from a minimum of three independent experiments.

Detection of DNA fragmentation

Total DNA was extracted using the DNAzol reagent, following the manufactor's instructions. DNA was solubilized in TE buffer and treated with RNAse (200μg/ml) for 2 hours at 37° C. Electrophoresis was performed on 1.5% agarose gels containing 1% ethidium bromide.

Cell cycle analysis

For analysis of cell cycle distribution $1x10^5$ cells were trypsinized, washed in PBS and fixed with ice cold ethanol (70%). After RNAse treatment (1mg/ml), cells were permeabilized with Triton X-100 and stained with propidium iodide (0.1mg/ml). To

determine the cell cycle distribution, DNA content was measured using a flow cytometer (FACScan).

DNA-PK activity assay

In order to assess DNA-PK activity, DNA-PK-dependent phosphorylation of a biotinylated p53-derived peptide (Glu-Pro-Pro-Leu-Ser-Gln-Glu-Ala-Phe-Ala-Asp-Leu-Trp-Lys-Lys. Promega) was measured in the presence of [^{32}P-γ]-ATP. Drug-treated or control cells were washed in low salt buffer (10mM HEPES, 25mM KCl, 10mM NaCl, 1.1mM $MgCl_2$, 0.1mM EDTA, 0.5mM PMSF, pH 7.2), pelleted and lysed by one freezing/thawing cycle as described in (21). After centrifugation at 10,000g for 5 minutes at 4°C, the supernatant was collected and used as cell extract. Protein content was determined using the Micro-BCA protocol (Pierce) with bovine serum albumin (Sigma) as standard. 10μg protein was incubated for 30 minutes at 30°C in DNA-PK reaction buffer (250mM HEPES (KOH, pH 7.5), 500mM KCl, 50mM $MgCl_2$, 1mM EGTA, 0.5mM EDTA, 5mM DTT), 0.025mM ATP, 0.5μCi [^{32}P-γ]-ATP, BSA 0.1mg/ml, and with human p53 oligopeptide as substrate in the presence or absence of activated calf thymus DNA. The final volume was 25μl. The reaction was stopped by addition of 25μl 30% acetic acid. 10μl was spotted on Whatman P81 membranes in duplicates and washed four times with 15 % acetic acid.

Membranes were placed on a phosphor imager screen for 2 hours. The screen was read on a phosphor imager (Storm 860, Molecular Dynamics) and the activity measured using the ImageQuant software package (Molecular Dynamics). Activity in the absence of

activated DNA was assumed to be unspecific and thus subtracted from corresponding measurements in presence of activated DNA.

Caspase-3 activity assay

For assessment of caspase-3 activity, cells were plated into culture dishes 24 hours before drug treatment. After drug treatment, cells were dislodged mechanically and washed twice in PBS. Caspase-3 activity was assessed as described by Enari et al. (22) with minor modifications: After five cycles of freezing and thawing in extraction buffer (50 mM PIPES-NaOH, pH 7.0, 50mM KCl, 5mM EGTA, 2mM $MgCl_2$, 1mM DTT, 20 μM cytochalasin B, 1mM PMSF, 1μg/ml leupeptin, 1μg/ml pepstatin A, 50 μg/ml antipain, 10 μg/ml chymopain) lysates were centrifuged at 10.000g for 12 minutes (4° C). The supernatant was immediately frozen in liquid nitrogen and stored at -80° C. Protein concentrations were determined using the Micro-BCA protocol (Pierce) with bovine serum albumin (Sigma) as standard. 36 μg protein were diluted in ICE standard buffer (100mM HEPES-KOH, pH 7.5, 10% succrose, 0.1% CHAPS, 10mM DTT, 0.1 mg/ml ovalbumin) containing the fluorogenic caspase-3 substrate DEVD-7-amido-4-methylcoumarin (DEVD-AMC, 1μM) and incubated for 30 minutes at 30° C. Fluorescence was measured using a fluorescence plate reader (Tecon, excitation 380nm, emission 460nm).

Immunoblotting

Cells were washed with PBS and lysed in RIPA buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1 % Nonidet P-40, SDS, 10 mM PMSF, aprotinin, sodium vanadate).

Protein concentrations were determined using the BCA protocol (Pierce) with bovine serum albumin as standard. 100 μg of protein were subjected to SDS gel electrophoresis (0.1% SDS/7.5% polyacrylamide) and blotted to PVDF membranes. After blocking with 5% skim milk in PBS, membranes were incubated with a polyclonal antibody against human DNA-PKcs (Santa Cruz Biotechnologies). A secondary HRP-conjugated antibody and the ECL Plus System (Amersham) were used for visualization. Chemoluminescence was measured using a Storm 860 Phosphorimager (Blue fluorescence, 650V, Molecular Dynamics).

Statistics

All data is presented as mean +/- standard error mean (SEM). A p-value <0.05 in a student t-test is proposed to be statistically significant.

Results

Inhibition of 26s proteasome function prevents radiation-induced G_2/M arrest

Ionizing radiation normally causes cells expressing wild type p53 to arrest in G1 phase through induction of the cyclin inhibitor p21. In cells with mutated p53, like PC3 prostate cancer cells, the situation can be more complex, but irradiation classically leads to G2/M arrest, irrespective of p53 status (reviewed in 23). The 26s proteasome controls the protein levels of p53, $p21^{WAF/CIP1}$, pRB, p27, cyclin A, B, and E. Thus, proteasome function is required for the transition from G1- to S-phase and late S- to G2/M-phase.

PC3 cells were treated with MG-132 (50 μM) for 3 hours prior to irradiation and then for a further 24 hours. Cells were stained with propidium iodide and analyzed on a flow

cytometer. The sub–G1 population increased 4-fold from initially 2.1 to 9.3 %, indicating apoptosis. Irradiation alone increased this population only slightly. The increase was additive with that induced by MG-132 (Fig. 1A/B). Additionally, we observed an increase in S phase cells, with up-to three peaks visible, in all cells treated with MG-132. This is interpreted as indicating that progression through this phase may be affected by proteasome inhibition and that PC-3 cells probably enter apoptosis from G1 or G2/M.

MG-132 reduces clonogenicity of cancer cells by induction of apoptosis

Since the effects of MG-132 are reversible, PC-3 and HD-My-Z cells were exposed to 50μM of drug for 3 hours, washed, trypsinized and subsequently plated into culture dishes to observe the impact on clonogenicity. Clonogenicity decreased by a highly significant amount in PC3 (64.59 ± 5.1 % to 35.4 ± 2.14, $p<0.001$ student-t) and HD-My-Z cells (14.69 ± 0.9 to 8.93 ± 1.27 %, $p< 0.01$, student-t) (Fig. 2A). A proportion of cells exhibited morphological criteria of apoptosis 24 hours after the start of incubation. To verify apoptotic death, DNA extracts of PC-3 cells were subjected to gel-electrophoresis. Treatment of PC-3 cells with 50μM MG-132 for 3 hours, followed by two wash-steps and incubation at 37° C for 24 hours, resulted in a 200bp DNA-laddering, typical for apoptosis (Fig. 2B).

MG-132 sensitizes cancer cells to ionizing irradiation

In order to examine whether inhibition of proteasome function alters the intrinsic radiosensitivity of cancer cells, PC-3 cells were exposed to 50 μM of MG-132. After 3 hours, PC-3 cells were washed, diluted, irradiated and plated into culture dishes. As

previously described for HD-My-Z Hodgkin cells (24). Inhibition of proteasome function sensitized PC3 cells to ionizing radiation (Fig. 2C).

MG-132 causes degradation of DNA-PKcs

Since proteasome inhibition induces apoptosis that is mediated by caspase activation, we considered the possibility that the observed radiosensitization was the result of destruction of the catalytic subunit of the DNA-PK complex, DNA-PKcs, which is required for repair of DNA double strand breaks. DNA-PKcs is a known substrate of caspase-3 (25). Permanent presence of MG-132 (50μM) in the growth medium of PC3 cells led to the appearance of the specific 160kDa degradation product of DNA-PKcs at 24 hours, which is the time that cells started to enter apoptosis. However, we could not detect the specific 160kDa fragment earlier or any significant changes in the levels of the intact 460kDa protein (Fig. 3A). The same results were obtained when MG-132 was washed out after 3 hours and cells lysates were studied after additional 24 hours of incubation (data not shown).

When DNA-PK activity was measured by phosphorylation of a p53 protein fragment in the presence of DNA double strand breaks, we found DNA-PK activity unchanged over a period of 5 hours after MG-132 treatment (Fig 3B). In order to exclude any interaction of of MG-132 with DNA-PK, lysates of PC-3 cells were incubated with MG-132 and studied for p53-phosphorylation. The drug did not decrease DNA-PK activity (Fig. 3C).

When caspase 3 activity was measured we were surprised to find a substantial drop in baseline caspase-3-like activity as early as 15' minutes after the end of MG-132 incubation. Combination of 30Gy irradiation with MG-132 pre-treatment also failed to

activate caspase-3-like activity. Caspase-3-like cleavage activity slowly started to recover 3 hours after the end of MG-132 treatment (Fig. 3D) supporting our observation that the sensitizing effect of MG-132 does not involve of caspase-3-mediated DNA-PK inactivation by DNA-PKcs cleavage.

Discussion

Specific inhibitors of 26s proteasome function induce apoptosis in a variety of cell lines (17, 20). Since the 26s proteasome is responsible for the degradation of pRB, p53, p21, p27, IκB, cFos, cJun and many other proteins essential for normal cellular function, this is perhaps not surprising. One of the most obvious effects of proteasome inhibition is on cell cycle distribution. In this report we confirm data presented in previous studies showing that proteasome inhibition blocks G1- to S-phase and S- to G2/M-Phase transition (26).

Additionally, our study shows that the plating efficiency of cells treated with this inhibitor for a short period of time is decreased. This loss of clonogenicity is most likely caused by apoptosis as cells showed morphological patterns of apoptotic cell death, an increase of the pro-G1 fraction in the FACS analysis, and typical 200bp DNA-laddering.

The mechanism that initiates apoptosis following proteasome inhibition remains unclear. Recent studies excluded any involvement of p53, the JNK kinase pathway or bcl-2 (19) and Bcl-X(L), Bax, Bad, Bak, or cytochrome c (27), which in part contradicts an earlier report (17). The apoptosis observed in this study after proteasome inhibition was independent of the p53 status as it was detected in PC-3 cells (p53 null) and HD-MyZ cells (mutated p53). NF-κB has been described as a possible survival pathway and

proteasome inhibition reduces NF-kB levels through increasing IκB, which is normally degraded by the 26s proteasome upon serine phosphorylation and subsequent ubiquitination. However, as we reported previously, NF-κB activity is not under control of IκBα in HD-MyZ Hodgkin's cells and proteasome inhibition leads to accumulation of IκBα without decreasing NF-κB activity (28), yet results in apoptosis. Wu and coworkers excluded the involvement of caspases in apoptosis of MO7e cells following proteasome inhibition, as caspase inhibitors failed to prevent DNA fragmentation (18). The critical pathway responsible for apoptosis following proteasome inhibition has therefore yet to be identified. It may depend on the cell type and the extent of inhibition. Like DNA-binding of the transcription factor NF-κB, proteasome function can promote but also inhibit cell death (29). For example thymocytes are rescued from apoptosis by proteasome inhibitors which indicate an active involvement of this protease in the cell death program (30). On the other hand proteasome inhibitors induce apoptosis in certain tumor cell lines (31, 32, 33, 27, 24, 35) and SV-40-transformed but not in normal human fibroblasts (35), supporting the view that proteasome function is essential for the survival of tumor cells. There are several possible explanations for the controversial impact of proteasome function on apoptosis in different cell types: (i) Cycling cells rely on controlled proteolysis of cyclins, pRb, p21 and p27. Accumulation of these proteins and subsequent cell cycle arrest in G1- and late S-phase might allow initiation of apoptosis more easily than in resting cells. (ii) Pro-caspase-3 was found to be present in the mitochondria in some cells. Its release into the cytosol was hypothesized to depend on mitochondrial PT pore opening mediated rupture of the mitochondrial outer membrane. This PT pore opening is prevented by cyclosporin, an inhibitor of the 26s proteasome, indicating

another possible link between proteasomes and apoptosis. (iii) The timing of proteasome inhibition during apoptosis might be critical as in thymocytes, rescuded from apoptosis by proteasome inhibitors, dexamethasone-induced apoptosis causes loss of proteasome function.

The importance of apoptosis in cell death following exposure to clinically relevant doses of ionizing radiation is controversial, but in general, the majority of cells in solid carcinomas do not enter the apoptotic death pathway. Instead, they undergo several cell divisions until they die or finally survive. Thus, the fate of cells in solid carcinomas after irradiation is rather determined by their ability to repair DNA damage caused by ionizing radiation than initiation of apoptosis (36). While single strand breaks (SSB's) are repaired relatively easily, the process of double strand break (DSB) repair is more complex and requires the presence and activity of the DNA-PK protein complex (37). Repetitious....Although NF-κB has been shown to have anti-apoptotic effects (38, 39, 40), no NF-κB dependent gene has been identified so far that controls DNA repair (For a review of NF-κB dependent genes see: 41). In a previous study we showed that down-regulation of NF-κB activity does not alter radiosensitivity in PC3 or HD-MyZ cells (28), although others have shown that it can have this effect in other cell lines. (I don't know. In this context I tried to make the link to DNA-repair.)

Proteasome inhibition sensitized PC-3 prostate cancer cells to ionizing radiation. Drug-induced changes in cell cycle redistribution are most unlikely to be responsible for this effect because MG-132 treatment mainly increased the number of cells in late S-phase, which has been shown to coincide with greatest radioresistance (42). As described previously for Lactacystein by Wu et al., proteasome inhibition by MG-132 can cause

degradation of DNA-PKcs, the catalytic subunit of DNA-PK (18) through a caspase 3 dependent pathway. Reduction of DNA-PK activity following inactivation or mutation of DNA-PKcs is known to result in enhanced radiosensitivity based on decreased repair of DNA-DSB's (43, 44, 45), although it is not clear whether this is a rate-limiting step in DNA repair that is affected by alterations in the level of DNA-PKcs. The level of DNA-PKcs expression has been reported not to correlate with radiosensitivity of gliomas (46). We were not able to detect meaningful changes in DNA-PK activity following MG132 drug treatment that did not seem to be a result of the apoptotic process. During 24hours of MG-132 treatment, immunoblotting with a specific antibody was unable to detect changes in either the intact 460kDa DNA-PKcs protein or the specific 160kDa product of caspase-3 cleavage. Only at 24 hours, when cells showed evidence of apoptotic cell death, was a specific 160kDa degradation fragment found. Additionally, we were not able to detect an early activation of caspase-3. but observed o substantial drop in DEVD-AMC cleavage activity.

According to the kinetics of the DSB-repair process described previously (47) any event interfering with the repair of DNA DSB's has to take place during the initial 6 hours after irradiation, which excludes the late degradation of DNA-PKcs observed in this study as a possible mechanism of radiosensitization.

The 26s proteasome has a fundamental role in cellular functions responsible for the control of protein levels, cell proliferation, immune response and signal transduction. Here we report an additional functional aspect of this protease in the response of human tumor cells to ionizing radiation, as we were able to link the control of its activity to DNA damage detection and/or repair. Although the mechanisms that initiate apoptotic

death and radiosensitization remain to be elucidated, proteasome inhibition presents as a promising tool for radiotherapy as it does not seam to act through sensitizing pathways utilized already or depends on the p53 status of tumor cells.

References

1. Björk-Eriksson, T; West, CM; Cvetskovska, E; Svensson, M; Karlsson, E; Magnusson, B; Slevin, NJ; Edström, S; Mercke, C. The lack of correlation between proliferation (Ki-67, PCNA, LI, Tpot), p53 expression and radiosensitivity for head and neck cancers. British Journal of Cancer, 80(9):1400-4, 1999
2. Gasinska, A; Urbanski, K; Jakubowicz, J; Klimek, M; Biesaga, B; Wilson, GD. Tumour cell kinetics as a prognostic factor in squamous cell carcinoma of the cervix treated with radiotherapy. Radiotherapy and Oncology, 50(1):77-84, 1999
3. Geiger, C; Weber, KJ; Wenz, F. Radiation induced chromosome aberrations and clonogenic survival in human lymphoblastoid cell lines with different p53 status. Strahlentherapie und Onkologie, 175(6):289-92, 1999
4. Caffo, O; Doglioni, C; Veronese, S; Bonzanini, M; Marchetti, A; Buttitta, F; Fina, P; Leek, R; Morelli, L; Palma, PD; Harris, AL; Barbareschi, M. Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. Clinical Cancer Research, 2(9):1591-9, 1996
5. Bonin, SR; Pajak, TF; Russell, AH; Coia, LR; Paris, KJ; Flam, MS; Sauter, ER. Overexpression of p53 protein and outcome of patients treated with

chemoradiation for carcinoma of the anal canal: a report of randomized trial RTOG 87-04. Radiation Therapy Oncology Group. Cancer, 85(6):1226-33, 1999

6. Hayakawa, K; Mitsuhashi, N; Hasegawa, M; Saito, Y; Sakurai, H; Ohno, T; Maebayashi, K; Ebara, T; Hayakawa, KY; Niibe, H. The prognostic significance of immunohistochemically detected p53 protein expression in non-small cell lung cancer treated with radiation therapy. Anticancer Research, 18(5B):3685-8, 1998

7. Pomp, J; Davelaar, J; Blom, J; van Krimpen, C; Zwinderman, A; Quint, W; Immerzeel, J. Radiotherapy for oesophagus carcinoma: the impact of p53 on treatment outcome. Radiotherapy and Oncology, 46(2):179-84, 1998

8. Rock, KL; Gramm, C; Rothstein, L; Clark, K; Stein, R; Dick, L; Hwang, D; Goldberg, AL. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell, 78(5):761-71, 1994

9. Rolfe, M; Chiu, MI; Pagano, M. The ubiquitin-mediated proteolytic pathway as a therapeutic area. Journal of Molecular Medicine, 75(1):5-17, 1997

10. Weissman, AM. Regulating protein degradation by ubiquitination. Immunology Today, 18(4):189-98, 1997

11. Hasselgren, PO; Fischer, JE. The ubiquitin-proteasome pathway: review of a novel intracellular mechanism of muscle protein breakdown during sepsis and other catabolic conditions. Annals of Surgery, 225(3):307-16, 1997

12. Tanaka, K. Molecular biology of proteasomes, Molecular Biology Reports. 21: 21-6, 1995

13. Stohwasser, R. and Kloetzel, P. M. Cytokine induced changes in proteasome subunit composition are concentration dependent, Biological Chemistry. 377: 571-7, 1996.

14. Nandi, D., Woodward, E., Gindburg, D.B., Monacco, J.J., Intermediates in the formation of mouse 20s proteasomes: Implications for the assembly of precursor ß subunits. EMBO J., 16, 101-113, 1997

15. Schimdt, M., Kloetzel, P.M., Biogenesis of eukaryotic 20s proteasomes: The complex maturation pathway of a complex enzyme, FASEB J., 11, 1235-43, 1997

16. Kloetzel, P.M., Soza, A., Stohwasser, R., The role of the proteasome system and the proteasome activator PA28 complex in the cellular immune response, Biol. Chem., 380, 293-7, 1999

17. Drexler HC: Activation of the cell death program by inhibition of proteasome function. Proceedings of the National Academy of Sciences of the United States of America 94:855-60, 1997

18. Wu, LW; Reid, S; Ritchie, A; Broxmeyer, HE; Donner, DB. The proteasome regulates caspase-dependent and caspase-independent protease cascades during apoptosis of MO7e hematopoietic progenitor cells. Blood Cells, Molecules, and Diseases, 25(1):20-9, 1999

19. Herrmann, J. L., Briones, F., Jr., Brisbay, S., Logothetis, C. J., and McDonnell, T.J. Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53, Oncogene. *17:* 2889-99, 1998.

20. Delic J, Masdehors P, Omura S, et al: The proteasome inhibitor lactacystin induces apoptosis and sensitizes chemo- and radioresistant human chronic lymphocytic

leukaemia lymphocytes to TNF-alpha-initiated apoptosis. British Journal of Cancer 77:1103-7, 1998

21. Ihara, M; Suwa, A; Komatsu, K; Shimasaki, T; Okaichi, K; Hendrickson, EA; Okumura, Y. Heat sensitivity of double-stranded DNA-dependent protein kinase (DNA-PK) activity. International Journal of Radiation Biology, 75(2):253-8. 1999

22. Enari M, Talanian RV, Wong WW, et al: Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380:723-6, 1996

23. Hwang, A; Muschel, RJ. Radiation and the G2 phase of the cell cycle. Radiation Research, 150(5 Suppl):S52-9, 1998

24. Pajonk, F., Pajonk, K. and McBride, W., Apoptosis and radiosensitization of Hodgkin cells by proteasome inhibition, submitted for publication, 1999

25. Teraoka, H., Yumoto, Y., Watanabe, F., Tsukada, K., Suwa, A., Enari, M., and Nagata, S. CPP32/Yama/apopain cleaves the catalytic component of DNA-dependent protein kinase in the holoenzyme, Febs Letters. *393:* 1-6, 1996.

26. Machiels, BM; Henfling, ME; Gerards, WL; Broers, JL; Bloemendal, H; Ramaekers, FC; Schutte, B. Detailed analysis of cell cycle kinetics upon proteasome inhibition. Cytometry, 28(3):243-52, 1997

27. Kitagawa, H; Tani, E; Ikemoto, H; Ozaki, I; Nakano, A; Omura, S. Proteasome inhibitors induce mitochondria-independent apoptosis in human glioma cells. Febs Letters, 1999 Jan 25, 443(2):181-6

28. Pajonk, F., Pajonk, K., McBride, W., Inhibition of NF-κB, Clonogenicity, and Radiosensitivity of Human Cancer Cells, JNCI, accepted for publication, 1999

29. Lin, KI; Baraban, JM; Ratan, RR. Inhibition versus induction of apoptosis by proteasome inhibitors depends on concentration. Cell Death and Differentiation, 5(7):577-83, 1998

30. Hirsch, T; Dallaporta, B; Zamzami, N; Susin, SA; Ravagnan, L; Marzo, I; Brenner, C; Kroemer, G. Proteasome activation occurs at an early, premitochondrial step of thymocyte apoptosis. Journal of Immunology, 161(1):35-40, 1998

31. McDade, TP; Perugini, RA; Vittimberga, FJ Jr; Callery, MP. Ubiquitin-proteasome inhibition enhances apoptosis of human pancreatic cancer cells. Surgery, 1999 Aug, 126(2):371-7.

32. Adams, J; Palombella, VJ; Sausville, EA; Johnson, J; Destree, A; Lazarus, DD; Maas, J; Pien, CS; Prakash, S; Elliott, PJ. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Research, 1999 Jun 1, 59(11):2615-22.

33. Zhang, XM; Lin, H; Chen, C; Chen, BD. Inhibition of ubiquitin-proteasome pathway activates a caspase-3-like protease and induces Bcl-2 cleavage in human M-07e leukaemic cells. Biochemical Journal, 1999 May 15, 340 (Pt 1):127-33.

34. Chandra, J; Niemer, I; Gilbreath, J; Kliche, KO; Andreeff, M; Freireich, EJ; Keating, M; McConkey, DJ. Proteasome inhibitors induce apoptosis in glucocorticoid-resistant chronic lymphocytic leukemic lymphocytes. Blood, 1998 Dec 1, 92(11):4220-9

35. An, B; Goldfarb, RH; Siman, R; Dou, QP. Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts. Cell Death and Differentiation, 1998 Dec, 5(12):1062-75.

36. Weichselbaum, RR., Little, JB, The differential response of human tumors to fractionated radiation may be due to a post-irradiation repair process, 1982, Br J Cancer, 46, p532-37

37. Jeggo, P. A. Identification of genes involved in repair of DNA double-strand breaks in mammalian cells, Radiation Research. *150:* S80-91, 1998.

38. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science, *281:* 1680-3, 1998.

39. Wu, M. X., Ao, Z., Prasad, K. V., Wu, R., and Schlossman, S. F. IEX-1L, an apoptosis inhibitor involved in NF-kappaB-mediated cell survival. Science, *281:* 998-1001, 1998.

40. Beg, A. A. and Baltimore, D. An essential role for NF-kappaB in preventing TNF-alpha-induced cell death. Science, *274:* 782-4, 1996.

41. May, M. J. and Ghosh, S. Rel/NF-kappa B and I kappa B proteins: an overview, Seminars in Cancer Biology. *8:* 63-73, 1997.

42. Sinclair, WK., Morton, RA, X-ray sensitivity during the cell generation cycle of cultured Chinese hamster cells, Radiat. Res, 29:450-474, 1966

43. Rosenzweig, KE; Youmell, MB; Palayoor, ST; Price, BD. Radiosensitization of human tumor cells by the phosphatidylinositol3-kinase inhibitors wortmannin and LY294002 correlates with inhibition of DNA-dependent protein kinase and prolonged G2-M delay. Clinical Cancer Research, 1997 Jul, 3(7):1149-56.

44. Chernikova, S. B., Wells, R. L., and Elkind, M. M. Wortmannin sensitizes mammalian cells to radiation by inhibiting the DNA-dependent protein kinase-mediated rejoining of double-strand breaks, Radiation Research. *151:* 159-66, 1999.

45. Hsieh, CL; Arlett, CF; Lieber, MR. V(D)J recombination in ataxia telangiectasia, Bloom's syndrome, and a DNA ligase I-associated immunodeficiency disorder. Journal of Biological Chemistry, 1993 Sep 25, 268(27):20105-9

46. Allalunis-Turner, MJ; Lintott, LG; Barron, GM; Day, RS 3rd; Lees-Miller, SP. Lack of correlation between DNA-dependent protein kinase activity and tumor cell radiosensitivity. Cancer Research, 1995 Nov 15, 55(22):5200-2.

47. Olive, PL; Banáth, JP; MacPhail, HS. Lack of a correlation between radiosensitivity and DNA double-strand break induction or rejoining in six human tumor cell lines. Cancer Research, 54(14):3939-46, 1994

Figure legends

Fig. 1

(A/B) MG-132 prevents radiation induced G2/M-arrest in PC-3 prostate cancer cells lacking p53 expression. Cell cycle distribution of PC-3 prostate cancer cells assessed by flow cytometry after staining with propidium iodide. Cells were pre-incubated with MG-132 (50μM) for 3 hours, irradiated and incubated for additional 24 hours in the presence of MG-132. Proteasome inhibition prevented the dose-dependent radiation induced G2/M-arrest, increased the number of cells in late S-phase as well as the pro-G1 population.

Fig.2

(A) MG-132 decreases clonogenicity of human tumor cell lines

PC-3 prostate cancer and HD-MyZ Hodgkin's cells were incubated with MG-132 (50μM) for 3 hours. Cells were washed twice and plated into culture dishes. After 14 days the colonies were fixed with ethanol, stained with cristall violett and counted. MG-132 treatment decreased the plating efficiency in all three cell lines.

(B) *Proteasome inhibition induced induces apoptosis in PC-3 prostate cancer cells*

PC-3 prostate cancer cells were incubated with MG-132 (50μM) for 3 hours, washed twice and incubated for additional 24 hours. Electrophoretic analysis of total DNA extracts (10μg) showed a 200bp laddering, typical for apoptosis.

(C) *MG-132 sensitizes human cancer cell lines to ionizing radiation*

PC-3 prostate cancer cells were incubated with MG-132 (50μM) for 3 hours, washed twice, irradiated and plated into culture dishes. After 14 days the colonies were fixed with

ethanol, stained with cristall violett and counted. MG-132 treatment sensitized PC-3 cells to ionizing radiation.

Fig. 3

(A) Degradation of DNA-PKcs by caspase-3 is a late event in proteasome inhibition induced apoptosis

Western blot of lysates from PC-3 prostate cancer cells after 0, 3, 6, 9, 24 hours incubation with MG-132 (50μM) using a specific antibody against human DNA-PKcs. The Antibody recognizes the intact 460kDa protein as well as the specific 160kDa fragment observed after caspase-3 dependent cleavage of DNA-PKcs. MG-132 caused no significant change of the DNA-PKcs protein levels over a period of 24 hours. Detection of a caspase-3-cleavage specific 160kDa fragment coincided with the occurrence of apoptosis.

(B) MG-132 does not decrease DNA-PK activity

PC-3 cells were incubated for 3 hours with MG-132 (50μM). Cells were washed and incubated at 37°C. At indicated times cells were lysed and DNA-PK activity was measured by phosphorylation of a human p53 derived oligopeptide in the presence of DNA DSB's. Proteasome inhibition did not cause any significant decrease of DNA-PK activity.

(C) *MG-132 does not interfere with DNA-PKcs directly*

Lysates of untreated PC-3 cells were incubated with MG-132. DNA-PK activity assay did not show any decrease DNA-PK activity excluding any direct interaction.

(D) MG-132 reduces constitutive caspase-3-like activity in PC-3 cells

PC-3 cells pre-incubated with MG-132 (50μM) for 3 hours, washed irradiated and incubated at 37°C. 15 minutes, 1 and 3 hours later cells were lysed and total cellular protein was assayed for caspase-3-like activity, measuring the release of AMC from the fluorogenic caspase-3 substrate DEVD-AMC. Proteasome inhibition completely inhibited constitutive caspase-3 activity after proteasome inhibition and combination with 30Gy ionizing radiation.